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The Effects of a Ketone Body on Synaptic Transmission

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The Effects of a Ketone Body on Synaptic Transmission

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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at the University of Kentucky

By

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ABSTRACT OF THESIS

The Effects of a Ketone Body on Synaptic Transmission

The ketogenic diet is commonly used to control epilepsy, especially in cases when medications cannot. The diet typically consists of high fat, low carb, and adequate protein and produces a metabolite called acetoacetate. Seizure activity is characterized by glutamate excitotoxicity and therefore glutamate regulation is a point of research for control of these disorders. Acetoacetate is heavily implicated as the primary molecule responsible for decreasing glutamate in the synapse; it is believed that acetoacetate interferes with the transport of glutamate into the synaptic vesicles. The effects on synaptic transmission at glutamatergic synapses was studied in relation to the ketogenic diet in *Drosophila* larvae for this thesis. Various measures of synaptic transmission were conducted. Acetoacetate decreased neurotransmission at the synapse. It was also found that acetoacetate has direct effects on the postsynaptic membrane, which indicates a novel role for the metabolite.

KEYWORDS: *Drosophila*, Acetoacetate, Glutamate, Excitotoxicity, Electrophysiology

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CHAPTER 1. INTRODUCTION

Historically, patients looked to fasting or water diets as a method of controlling seizures (6). In the 1920's, a high fat, low carb diet, otherwise known as the ketogenic diet, was found to mimic the effects of those kinds of diets (6). The synthesis of ketones was implicated in producing these effects with the high fat diet (6). The ketogenic diet has been shown to be efficacious as a method for seizure reduction in children (1,2), with many of them being able to discontinue the diet after a period and remain seizure-free or seizure-reduced (3), which may point to the diet altering the brain in a long-lasting way.

Epilepsy has an approximate prevalence of 1.54% in rural areas and 1.03% in urban areas (4). Medications for the treatment of epilepsy are effective for many patients and there are a diverse number of options; however, refractory epilepsy is noted in approximately 1/3 of this population (5). Refractory epilepsy is defined as epilepsy that is unable to be controlled by traditional medications (5). The ketogenic diet has been shown to be efficacious in the treatment of refractory epilepsy (1,2). Additionally, there is a noted treatment gap for this population that may range from 10% to 75% depending on the population. This treatment gap is largely due to a lack of access or a lack of ability to afford medication (4). Both of these factors point to a need for an alternative form of treatment.

The Johns Hopkins protocol for implementation of the diet is widely used. The patients start with a self-imposed reduction in carbohydrate intake followed by a small fast. Upon admittance to a hospital, blood glucose levels are monitored and the patient begins the ketogenic diet under the supervision of a physician. The protocol calls for the

use of eggnog as the source of fat for the first two and a half days. At the end of the third day, the patient tries a full ketogenic meal and tolerance, or lack thereof, to the meal determines if the patient can continue on with the protocol. If the patient tolerates this meal and a follow up meal the next day, they can be discharged and will continue the diet on their own (7). A typical ketogenic diet consists of a 4:1 ratio of fats to carbohydrates and protein combined. Total daily calorie needs are calculated based on standard parameters for caloric intake, like height, weight, age, BMI, and activity level (12).

There has been evidence for a modified-Atkins approach to controlling seizures. A modified-Atkins diet features the same high fat, low carbohydrate intake. It primarily differs in that the patient also eats high levels of protein. One major problem with the ketogenic diet is the side effects. These side effects can be severe and can lead to a lack of compliance with the diet. A modified-Atkins diet has not only been shown to be just as efficacious, but patients reported less severe side effects and increased satiety with the diet, which can help increase compliance of the patient (8,10,11).

Adherence to the diet is a major concern for health care providers due to the symptoms many patients will experience. The top two reported side effects include dehydration and gastrointestinal discomfort. Less common, but serious, side effects include renal stones, cardiomyopathy, and anemia; these are often characterized by a later onset than the more commonly seen ones (9). Inadequate growth has been noted in some populations. Often, nausea is reported with the onset of the diet (7).

The type of fat ingested with a ketogenic diet may play a role in the efficacy of the diet in reducing seizures. Traditionally ketogenic diets are characterized as a diet composed primarily of long-chain triglycerides. There is metabolic evidence for a diet

primarily composed of medium chain triglycerides rather than long chain triglycerides. It has been shown that medium-chain triglycerides are more ketogenic than long-chain triglycerides due to differences in how the triglyceride is handled by cells (7). Medium-chain triglycerides are known to supply intermediates for citric acid cycle (7) and may even allow for a slightly higher carbohydrate and protein intake for the patient (12). However, a medium-chain triglyceride ketogenic diet is associated with increased levels of gastrointestinal distress (12). Studies have shown that a ketogenic diet composed primarily of medium-chain triglycerides is as effective in seizure reduction as a diet composed of long-chain triglycerides (50, 51, 52).

Another variation on the ketogenic diet is the low-glycemic index ketogenic diet. This diet also allows for higher carbohydrate intake, at 40-60 grams per day. All carbohydrates ingested must score a 50 or lower on the glycemic index (13). The glycemic index is a measure of the quality of the carbohydrate; high glycemic index carbohydrates elicit a stronger insulin response and cause a larger spike in blood glucose levels (14). This diet was also shown to be efficacious for refractory epilepsy (15,16,13).

The need for an alternative treatment method is warranted not only by its efficacy, but by the lack of efficacy and adherence to traditional treatment methods. Several classes of anti-epileptic medications exist, each with their own problems and intricacies. Suicidal ideation is a known problem for patients on anti-epileptic drugs, with a calculated odds ratio 3.53 compared to a placebo (17). Nonadherence to these treatments is also an issue (17,18); one study found a non-adherence rate that was approximately 30% (18).

Seizure activity is characterized as synchronized neuronal discharge in the brain in excess (19,20). Signs and symptoms of a seizure can vary, but typically include shaking

of the body and certain limbs. Epilepsy is defined as a propensity to display repeated seizure activity (19). Seizures are often classified according to the location of origin of the neuronal discharge; they are sometimes further characterized by the consciousness status of the patient during the seizure as well as motor involvement or lack thereof (21). Epilepsy can be divided into either focal epilepsy or generalized epilepsy. Generalized epilepsies involve both hemispheres of the brain whereas focal epilepsies have a specific origin point and may spread thereafter.

Excitatory neurotransmission in the normal brain relies heavily on the neurotransmitter glutamate. Seizure activity causes an elevation of levels of glutamate in the brain, leading to excitotoxicity. Excess glutamate at the level of the synapse causes many morphological and signaling changes, often which result in pre- and post-synaptic changes that lend themselves to further seizure activity. These changes promote future hyperexcitability in response to glutamate via plasticity (23).

Glutamatergic derived excitotoxicity is a direct result of the overload of glutamate in a synapse; this pathological state is implicated in many other disorders of the brain that involve elevated levels of glutamate. Excitotoxic injury is characterized primarily as the detrimental effects that arise due to an aberrant rise in intracellular calcium levels (24, 43).

The elevation in intracellular calcium levels arises, in part, via certain glutamate receptors on the postsynaptic membrane. Glutamatergic NMDA receptors allows flux of calcium ions upon ligand binding (24). AMPA receptors allow sodium flux; if the GluR2 subunit is not present in the channel, then calcium can flux through as well. (25, 26, 27). Kainate-type receptors typically do not allow for the flux of calcium (24). Excess

glutamate in the synapse may cause an increase in the amount of time that NMDA and some AMPA receptors are allowing ion flux, resulting in increased intracellular calcium. Additionally, metabotropic glutamatergic receptors (mGluRs) play a role in the rise in intracellular calcium levels. Group 1 mGluRs include mGluR1s and mGluR5s (28, 29). These mGluRs are found on both the pre- and post-synaptic membrane (28, 29). On the postsynaptic membrane, they are found to potentiate ion flux through NMDA receptors, further contributing to the rise in intracellular calcium (30).

While a rise in intracellular calcium is primarily implicated as the pathological condition responsible for excitotoxicity, it doesn't alone explain how apoptosis or necrosis is induced. There is evidence for mitochondrial involvement post rise in intracellular calcium. The mitochondria sequester the calcium out of the cytoplasm, causing mitochondrial damage (31, 32). In addition to this mitochondrial damage, nitric oxide synthase and calcium-mediated proteases are activated (33, 24, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43), which all can give rise to cellular destruction.

Fatty acids are broken down in the body via beta-oxidation, which produces acetyl CoA. With a ketogenic diet, levels of acetyl-CoA are elevated beyond what can be used by the Krebs Cycle in this state. Oxaloacetate is preferentially utilized in gluconeogenesis, which downregulates flux through the Krebs Cycle. While the preferred metabolic substrate of the human body is glucose, the very low carbohydrate intake associated with the ketogenic diet forces the body to preferentially break down triglycerides in order to maintain necessarily levels of ATP production. The liver shunts this excess acetyl-CoA to a different metabolic pathway because it lacks to ability to metabolize it. The ketone bodies (acetoacetate, beta-hydroxybutyrate, and acetone) are

primarily formed in the hepatic mitochondrial matrix from this excess acetyl-CoA (49). These ketone bodies are known to be able to cross the blood-brain barrier, which is why they have been a point of research in neuromodulation (44, 45, 46).

Acetoacetate, a weak acid, is formed via the decarboxylation of a carboxyl group of acetoacetic acid (47, 48). It carries a formal charge of -1 (47). Its predicted water solubility is 240g/L (48).

1.1 Potential mechanisms of action for ketone bodies

Ketone bodies have been implicated in a number of cellular mechanisms; relevant potential mechanisms as they relate to seizure activity are discussed here. It is hypothesized that ketone bodies may act in a manner that promotes "reduced glucose utilization/glycolysis, reprogrammed glutamate transport, indirect impact on ATP-sensitive potassium channel or adenosine A1 receptor, alteration of sodium channel isoform expression, or effects on circulating hormones including leptin" (49).

One working model for the modulatory effects of the ketogenic diet on seizure activity revolves around changes in the biosynthesis of glutamate or GABA, the major inhibitory neurotransmitter. Glutamate is released into a synapse and subsequently taken up by astrocytes, where it is converted into glutamine via glutamine synthetase. Following this, the glutamine is transported to neurons where it is converted back to glutamate. Glutamate can be converted into GABA or into aspartate, the latter of which is a transamination reaction that requires the substrate oxaloacetate. Oxaloacetate is an intermediate in the Kreb's cycle; flux through the Kreb's cycle is known to be increased with the ketogenic diet. This increased pathway flux would sequester oxaloacetate,

allowing for less conversion of glutamate into aspartate; glutamate to GABA conversion is upregulated. Upregulation of GABA increases the prevalence of an inhibitory neurotransmitter, which could potentially modulate the hallmark excitatory neurotransmission seen with seizure activity (55,56).

Experimental evidence for this theory provides some support, yet lacks being substantiated enough to be generally accepted for the mechanism to be effective at reducing seizure activity. One study showed the ability of a ketogenic diet to alter levels of aspartate, yet no changes in levels of glutamate or GABA were able to be shown (55,56). Another study performed in rats provided evidence for a reduction of glutamate levels, yet there was no significant change in levels of GABA (57,55). Analysis of cerebrospinal fluid from children on ketogenic diets show increase levels of GABA, but no change in glutamate concentrations (58,55). Elevated levels of glutamine and glutamate have been shown in the hippocampus of rats fed a ketogenic diet (59,55). A different study showed increased levels of mRNA of the GABA synthesizing enzyme glutamic acid decarboxylase, but no increase in GABA levels (60,55).

A different theory used to explain the effects of the ketogenic diet in the context of modulating excitotoxic synaptic transmission relies on the activation of an ATP-sensitive potassium channel. It was shown that acetoacetate and beta-hydroxybutyrate were modulating the firing of GABAergic neurons through the K(ATP) channel (61,55). However, it is known that this potassium channel closes with higher levels of ATP; ATP levels are upregulated with the ketogenic diet. ATP-mediated closure of these potassium channels should increase, rather than decrease, neuronal excitability (62,55).

Another theory for the modulatory effects seen with the ketogenic diet is the upregulation of the inhibitory modulator adenosine. The adenosine A1 receptor is found on excitatory neurons; adenosine binding produces an inhibitory effect in these neurons. Adenosine biosynthesis and concentration levels is reflective of levels of ATP; ATP levels are upregulated with the ketogenic diet (63,64,55). Evidence for this theory comes from a study in which heterozygous knockdowns of the receptor, homozygous knockouts of the receptor, and upregulation of adenosine kinase induced seizure activity. For both the heterozygous knockdown group and the adenosine kinase overexpression group, seizure activity could be modulated with the ketogenic diet. The homozygous receptor knockout did not show this same effect (65,55).

A different theory focuses on changes in mitochondria seen with the ketogenic diet (66,55). The ketogenic diet upregulates production of enzymes involved in mitochondrial metabolism and the biosynthesis of mitochondria themselves (59,55). The ketogenic diet is also known to reduce oxidative stress through different mitochondrial-based mechanisms, including increasing levels of glutathione, a known antioxidant. Glutathione levels are reduced in epilepsy (67,55). The ketogenic diet was also shown to increase levels of NF E2-related factor 2 (Nrf2), a transcription factor. Nrf2 modulates transcription of genes involved in the glutathione pathway of mediating oxidative stress; Nrf2 itself is activated by cellular stress (68,55). It was shown in mouse models that an increase in Nrf2 decreases spontaneous seizure activity (69,55).

Ketone bodies were shown to be able to increase the activity of catalase in response to hydrogen peroxide as well as increase oxidation of NADH, thereby reducing reactive oxygen species (ROS) levels (70,71,55). Several studies have shown that ketone bodies

reduce mitochondrial ROS (72,73,55). This decrease in ROS may be due to an increase in expression of uncoupling proteins. Uncoupling proteins can alter the proton gradient generated in mitochondria. Increasing their activity decreases the mitochondrial membrane potential, which, in turn, decreases ROS production. Resistance to induced seizure activity through this mechanism has been shown (74,76,55). Fatty acids can increase levels of certain transcription factors, such as proliferator-activated receptor, or the forkhead box. These transcription factors can upregulate expression of uncoupling proteins, which can cause mitochondria dysregulation (75,55).

One study showed beta-hydroxybutyrate to be an inhibitor of class I histone deacetylases. Beta-hydroxybutyrate increases acetylation of lysine 9 and lysine 14 on histone 3 and increases transcription of enzymes that function as antioxidants. This inhibition was shown to provide resistance to oxidative stress. However, the evidence for the role of beta-hydroxybutyrate in this manner has yet to be shown in a context related to neural activity (77,55).

A different theory relies on the fact that an increase in neurotransmission, which is a hallmark characteristic of seizures, would decrease levels of certain Kreb's cycle intermediates. One of these intermediates, alpha-ketoglutarate, also functions as a precursor molecule in the biosynthesis of glutamate (and GABA). The increase in neurotransmission would deplete levels of ATP production by decreasing levels of acetyl CoA, another intermediate involved in the Kreb's cycle. The ketogenic diet is known to both increase ATP levels and provide intermediates for the Kreb's cycle; the ketogenic diet also decreases flux through glycolysis in favor of flux through alternative metabolic pathways involving ketone bodies (78, 62,79,80,55).

Glycolytic inhibitors have been a focus of drug research. In hippocampal slices, an inhibitor of phosphoglucose isomerase, an enzyme involved in glycolysis, was shown to be able to diminish excitatory neurotransmission that was invoked via 4-aminopyridine, a potassium channel blocker (81,55). A similar effect was shown using the same inhibitor in mice and rats (81,82,83,55). Additional evidence for glycolytic inhibitors as a potentially fruitful target for modulation of seizure activity is seen in studies utilizing Fructose 1,6 bis-phosphate, which decreases flux through glycolysis and increases flux through the pentose-phosphate pathway. Studies have shown this compound to be effective in reducing invoked seizure activity (84,55).

Another theory relies on modulation through polyunsaturated fatty acids (PUFAs). PUFAs are fatty acids in which there are multiple sites of unsaturation within the fatty acid chains. The ketogenic diet has been shown to increase the mobilization of PUFAs from adipose tissue to the brain and liver (85,55). A standard ketogenic diet does not typically include high amounts of PUFAs, which indicates that this change in PUFA levels throughout the body is an endogenous response to the diet rather than a byproduct of the increased fat intake (86,55). PUFAs have been shown to act on voltage-gated sodium and voltage-gated potassium channels, two types of ion channels involved in neurotransmission (87,88,55). PUFAs can also activate a nuclear receptor, peroxisome proliferator-activated receptor-alpha, which is known to regulate the transcription of many genes involved in metabolism (89,55). There have been mixed results in the efficacy of PUFA supplementation. One study showed that a ligand for peroxisome proliferator-activated receptor-alpha had anti-seizure effects (90,55). Other studies have

shown there to be no effects of seizure activity while others have shown only a transient effect (91,92,55).

Another theory for the modulatory effects seen involves the free fatty acid receptor 3 (55). Short-chain fatty acids are ligands for the receptor and activation of the receptor will cause inhibition of the N-type voltage-gated calcium channel. One study has shown beta-hydroxybutyrate to be an agonist for this receptor (93,55). However, a different study showed it to be antagonistic to this receptor, making the evidence for this theory largely inconclusive. Furthermore, the studies did not characterize the potential effect this may have on the characteristic neurotransmission seen with seizure activity (94,55).

A final potential mechanism of action involves modulation through VGLUTs. VGLUTs are the transporters involved in loading glutamate into a vesicle in a presynaptic neuron. The release of neurotransmitters into the synapse is highly regulated and most aspects have been rigorously characterized prior. A simplistic way of approaching the modulation of release of the neurotransmitter would be to consider the two main aspects involved: the number of vesicles being exocytosed in response to a stimuli and the amount of neurotransmitter contained within those vesicles. VGLUT modulation would primarily affect the latter. A compelling theory for modulation via these VGLUTs relies on allosteric modulation via a chloride binding site on the VGLUT itself. Through this chloride binding site, the amount of neurotransmitter being packed into vesicles can be affected (53,54,55).

1.2 Summary of Project Aims

With this project, I aimed to explore the potential modulatory effects that a ketogenic diet may have on neurotransmission. I accomplished this in two primary ways: through feeding *Drosophila* larvae a ketogenic diet and through direct application of the ketone body of interest, acetoacetate, on semi-intact preparations. Measures of synaptic transmission were primarily taken through electrophysiology methods, which are described in great detail in the methods section of this thesis. The working hypothesis for this project was that both the ketogenic diet and direct application of acetoacetate will modulate neurotransmission in a way that is consistent with a decrease in transmission. The following aims were addressed within this project:

- Determine the effects of a ketogenic diet on synaptic depression
- Determine the effects of acetoacetate on synaptic depression
- Determine the effects of acetoacetate on spontaneous quantal events
- Determine if there is evidence for a distinct pool of vesicles for spontaneous quantal events

Acetoacetate was chosen as the ketone body of interest due to previous literature (56) finding that is had the strongest effect on transmission. The model used, the larval *Drosophila* neuromuscular junction, lends itself well to a project like this because it is a glutamatergic synapse that is easily identified and indexed for synaptic transmission. Additionally, the dissection is a semi-intact preparation, giving more physiological relevance than some other preparations. A more rigorous argument for the larval *Drosophila* NMJ will be presented later in this thesis.

CHAPTER 2. BACKGROUND

2.1 Previous findings on the modulatory effects of acetoacetate on the loading of synaptic vesicles

Each synaptic vesicle must be packaged with the desired neurotransmitter in order to have neurotransmitter released into the synapse. The process of packaging glutamate into vesicles relies on several different pumps and channels embedded in the vesicle membrane. An ATPase generates an electrochemical gradient by pumping protons into the vesicle. This electrochemical gradient drives the movement of glutamate through the VGLUT. Uptake of glutamate also displays a chloride dependence. Chloride ions, which carry a negative charge, enter into the vesicle and offset the positive charge generated by the proton pump. This offset is essential, because it allows for the buildup of protons, which creates a pH difference from the outside of the vesicle, while still maintaining the membrane potential of the vesicle itself. However, this effect is primarily seen at high chloride concentrations. At physiological conditions, chloride is often sequestered; intravesicular concentrations of chloride will already be high. There is evidence that chloride already located in the vesicle may serve as a counter-transport ion for the transport of glutamate, either through the VGLUTs themselves or even through the chloride channel. A more interesting piece of evidence is the activation of glutamate transport that is seen with chloride concentrations in the millimolar range (53). At low concentrations of chloride, uptake is low. This uptake of glutamate is drastically increased with an increase of chloride to a concentration of 4mM. At higher concentrations of chloride, the uptake of glutamate into vesicles is actually inhibited (54).

This activation and then deactivation is not explained by the mechanisms described above; it points to an allosteric modulatory binding site for chloride (53,54).

This allosteric binding site was confirmed by Juge and colleagues in 2010 using a purified VGLUT transporter inserted onto an artificial vesicle membrane. They showed a lack of chloride transport through the VGLUT itself, but a dependence of the VGLUT on chloride concentrations, indicating allosteric regulation. Furthermore, they explored over 30 different metabolites for their ability to modulate this glutamate transport. Acetoacetate, beta-hydroxybutyrate, and pyruvate were all found to inhibit this glutamate transport. Pyruvate is an intermediate in glycolysis. Off all compounds tested, they found acetoacetate to provide the strongest inhibitory effect (54).

Juge et. al. found that application of acetoacetate changed the required concentrations of chloride for equivalent glutamate uptake as determined by previous assays. Essentially, a higher chloride concentration was required in order to get sufficient uptake of glutamate through the VGLUT. This indicates that acetoacetate is a competitive inhibitor of chloride activation of the VGLUT. They did find that acetoacetate could be washed out without long-lasting effects (54).

Juge and colleagues went on to investigate the effects of acetoacetate on glutamate release *in vivo*. Glutamate release from hippocampal-derived neurons and astrocytes was studied. Astrocytes are known to take up glutamate and store it in microvesicles; they also express VGLUTs. They found that acetoacetate could inhibit glutamate release from the neurons but not the astrocytes, suggesting specificity of the ketone body for neuronal-derived vesicles. This was assayed in manner in which glutamate release was evoked (54).

When considering modulatory effects of a substance on neurotransmission, it is important to consider both presynaptic and postsynaptic effects. Investigating vesicular glutamate release is primarily a presynaptic effect. One way in which postsynaptic effects can be characterized is through spontaneous quantal events. Spontaneous quantal events are non-evoked events in which a single quantum of neurotransmitter is released into the synapse and a small, graded response is seen in the postsynaptic cell. These events are helpful in characterizing the potential effects of a substance on various aspects of the postsynaptic cell, such as receptors. If a substance were to be also modulating the postsynaptic receptors, either through binding to the receptor itself or through a mechanism in which the number of receptors are downregulated, then the characteristics of these spontaneous quantal events would reflect that. Often, effects on the postsynaptic target can be masked with evoked stimulation; pairing data on spontaneous quantal events with data on evoked events may give a more complete picture of the modulatory effects of a substance (54).

Juge reported a decrease in the average amplitude and frequency of these spontaneous quantal events with the application of acetoacetate; this was done on mouse hippocampal slices. They also did a paired pulse ratio and determined that there was no change in the probability of release. Taken together, Juge and colleagues concluded that there was evidence for a decrease in the relative packaging of glutamate. However, they were unable to definitively determine that the changes seen with the decrease in frequency and amplitude of the quantal events were solely due to a decrease in packaging rather than postsynaptic effects. Finally, Juge and colleagues were able to show a decrease in seizure-like activity, induced in rats through administration of 4-

aminipyridine, with acetoacetate (54). The information derived from their research determined the working theory that the research presented in this thesis is derived from.

2.2 Vesicle pool recycling

Vesicles within the presynaptic neuron are recycled. Post exocytosis of their contents, they are refilled with neurotransmitter. There has been much evidence for distinct pools of vesicles within the presynaptic terminal bouton, but there has yet to be conclusive biochemical analysis confirming the exact differences. Most of the literature has already adopted the pool terminology and the likelihood, based on numerous observations, that there are multiple pools (95).

The readily-releasable pool is typically defined as the pool from which vesicles are utilized quickly in response to stimulation. The reserve pool is typically defined as the pool from which vesicles are drawn from in the context of longer, sustained stimulation (95). Observations that there is a difference in the capability of different vesicles that are not in the readily releasable pool to release their context may indicate that there are 3 distinct pools (96,95). Often, this third pool is called the recycling pool (95).

The readily-releasable pool consists of the vesicles that are quickest to exocytose their contents upon stimulation (95). They are docked in the active zone of the presynaptic neuron and they can be depleted in as quick as a few milliseconds (97,98,95). The readily-releasable pool makes up only a small fraction of vesicles in the terminal bouton. The recycling pool consists of vesicles that are released with more moderate stimulation (95). At physiological conditions, it has been shown that this pool is continually recycled (99,100,101,102,95). This pool may contain anywhere from five to

twenty percent of vesicles found in the terminal bouton. Finally, the reserve pool contains the vesicles that are only released upon prolonged, intense stimulation. It may contain up to 90 percent of vesicles found in the terminal bouton. It is thought that the reserve pool may be utilized post depletion of the recycling pool, though there has yet to be a molecular mechanism identified for this (103,104,105,95).

The *Drosophila* neuromuscular junction (NMJ) contains approximately 84,000 vesicles (106,95). There are about 300 vesicles in the readily-releasable pool, about 14,000 vesicles in the recycling pool, and about 70,000 vesicles in the reserve pool (95). The NMJ of *Drosophila* exhibits both a fast and slow recycling of vesicles (107,108,109,110,95). It is thought that the reserve pool may arise from bulk endocytosis of the plasma membrane while the recycling pool is formed from direct endocytosis of the plasma membrane (95). It is also been proposed that there is an even fast method of recycling, termed the "kiss and run" method, that some vesicles in the readily-releasable pool may utilize. This method involves transient fusion with the plasma membrane and subsequent pinching off of the vesicle, rather than integration of the vesicle with the plasma membrane (111,112,95).

Currently, there is some debate as to where the vesicles used for spontaneous quantal events are coming from. Some studies have shown these vesicles to be drawn from one of the three known pools described above. Other studies have provided compelling evidence that there may be a distinct pool of vesicles that are reserved for these spontaneous quantal events (126,127,128,129). One aim of this project is to try and address this controversy.

2.3 Drosophila NMJ as a model

Any model utilized should possess the necessary features of the disease to be studied. Seizure activity is characterized as primarily a glutamatergic response in the brain. Larval *Drosophila* NMJs exclusively utilized ionotropic GluRs (113,114). In terms of homology, they are closed to the human AMPA glutamatergic receptor. Their postsynaptic densities resemble densities found in mammals. The ideal model should also be simple enough that results can be inferred without being too simple that the complexity found within the normal disease state is lost. *Drosophila* NMJs are a simple system that has been well characterized; their NMJs are easily visualized and recorded from with great accuracy. Their NMJs showed reliably characteristic connectivity while still maintaining plasticity throughout development and in response to activity. Additionally, there is evidence of synaptic refinement at the NMJ (113).

The larval NMJ has three different types of boutons innervating the NMJ. The bouton type of interest for this project is Type I. Type I boutons are glutamaterigic and are typically sybdivided into Is and Ib, based on size. There are anywhere from 20-60 of these type I boutons on each muscle in *Drosophila* larva. It is typical of studies to look at muscles 6 and 7; this NMJ is innervating two muscles, so it has twice as many type I boutons (113). The larval *Drosophila* NMJ is ideal for studying because it produces graded responses in the post synaptic membrane rather than action potentials. While action potentials are considered to be “all-or-none” and typically have an amplitude that does not vary, graded excitatory junction potentials (EJPs) do vary in amplitude. The variation in amplitude can be correlated to the amount of neurotransmitter being released into the synapse, which makes it ideal for studying modulation of vesicle turnover (123).

Postsynaptic glutamatergic receptor subunit composition can vary among different receptors, but it typically heterotetrameric. Known possible subunits include GluRIIA, GluRIIB, GluRIII, GluRIID, and GluRIIE (114,115). GluRIII is required for transmission; knockouts are lethal. GluRIID and GluRIIE appear to be essential for transmission; without them, there is no ionic current through the receptor. The flexibility within subunit composition lies within GluRIIA and GluRIIB; at least one of these is needed for correct receptor formation, but it does not appear to matter which one is selected. Receptors containing the GluRIIA subunit exhibit slow desensitization while GluRIIB promotes fast desensitization (114, 115,130). The overall balance between the number of these two subunits present in a population of glutamatergic receptors may influence the strength of transmission seen (114,130).

Although the glutamatergic receptors found at larval *Drosophila* NMJs are primarily considered to be homologous to AMPA receptors, there has been pharmacological evidence that they may distinct from AMPA receptors. Studies have shown that the glutamatergic receptors are not responsive in the expected way to both AMPA and Kainate, which are the two primary agonists against the receptors of the same name, respectively. Instead, it has been proposed that the larval Drosophila NMJ glutamatergic receptors are of a Quisqualate subtype rather than an AMPA subtype (115). This piece of evidence is important and must be taken into consideration when interpreting the results electrophysiological studies on the NMJ of *Drosophila*.

The larval *Drosophila* NMJ is the best model to study potential effects of a modulator on glutamatergic-based synaptic transmission because glutamate is utilized here apart from other neurotransmitters. One could argue that the lack of inhibitory

neurotransmission, especially from GABA, could render this model to simplistic to provide translational results. However, there is strong evidence for the GABA receptor internalization with seizure-like activity, making GABA neurotransmission a less relevant part of the equation (116,117,118,119). The primary interest of this study is understanding the modulatory effects of ketone bodies on glutamatergic transmission; the best model in which to do this would be a model in which glutamatergic transmission stands alone because the results can be interpreted without ambiguity and without confounding factors

CHAPTER 3. METHODS

3.1 Introduction

Larval *Drosophila* serve as an ideal model organism for electrophysiology studies on glutamatergic synapses because their NMJ utilizes glutamate apart from other neurotransmitters. This enables results to be interpreted without ambiguity. Their NMJs are easily accessible and recorded from; preserved homology of the NMJ profile allows for results that are translatable. The postsynaptic muscle produces graded EJPs, allowing for the amplitudes to be analyzed. The brevity of the *Drosophila* life cycle makes it a desirable preparation. The life cycle of the larvae persists in stages, each with hallmark differentiation characteristics, which allows for staging based on lifespan. Prior electrophysiological studies have established well defined parameters for acceptable measures for this preparation. The methodology presented in this section can be broken up into two major categories: general housekeeping and methodology that is preserved across all experimental setups, unless otherwise noted, and specific methodology used for each major experimental protocol that was utilized.

3.2 Methods and Materials

3.2.1 Fly Stock Maintenance

Canton-S (CS) *Drosophila melanogaster* have been kept isogenic in the lab for a number of years and were originally obtained from the Bloomington Fly Stock. They are

raised on a standard cornmeal diet and kept on 12-hour light dark cycles. They are housed at 21 - 23 degrees Celsius. General staging and development cycles of *Drosophila* has been previously described (120), but will be summarized in brief below.

Drosophila larvae progress from first through third instar staging. Each stage exhibits characteristic details, such as changes in the morphology of the mouth hooks, the size of the larvae, and the development of the sphericals. These morphological markers allow the larvae to be accurately grouped based on current age. Egg-pulse chambers are utilized for additional accuracy in staging. With these chambers, eggs are collected within a four-hour window and then housed separate. This ensures that all flies utilized were laid within a narrow range of time. *Drosophila* are housed in vials partially filled with a cornmeal-agar-dextrose-yeast medium; the exact contents of this diet have been described prior (121).

3.2.2 Pharmacology

The preparations are maintained in the hemolymph-like saline 3 (HL3) [in mM: 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 25 5N,N-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES)] at a pH of 7.1 (131). This saline has shown to be the most optimal option for preserving dissected larvae. 10mM Lithium Acetoacetate (Sigma Aldrich) (LiAA) was mixed into the HL3 saline upon use on experimental days.

3.2.3 Electrophysiological recordings

A semi-intact preparation was utilized for electrophysiological recordings. The cuticle of the larvae is pinned on either end and a longitudinal incision is made on the dorsal side. The cuticle is laid flat and internal organs are removed to reveal the body wall muscles. The brain of the larvae is removed by cuts made underneath and around it, freeing it from the connecting tissue. All segmental nerves are cut from the brain as close the brain as possible, preserving the segmental nerve as well as its connection onto muscle (the NMJ) (124).

Electrophysiological recordings were made from muscles 6 or 7 of segment three or four. All recordings were taken immediately post dissection. All preparations were dissected as 3rd instars. Recordings were made at room temperature (20 – 21 degrees Celsius) utilizing standard procedures (115). Postsynaptic recordings were taken with a sharp glass intracellular recording electrode filled with 3M KCl. An AxoClamp-2 B amplifier was utilized to visualize the results. A glass suction electrode was used to capture the cut end of the appropriate segmental nerve. Stimulatory electrical signals were made with a Grass S88 dual stimulator. The PowerLab/4s interface was used to record signals to a computer (Figure 3.1).

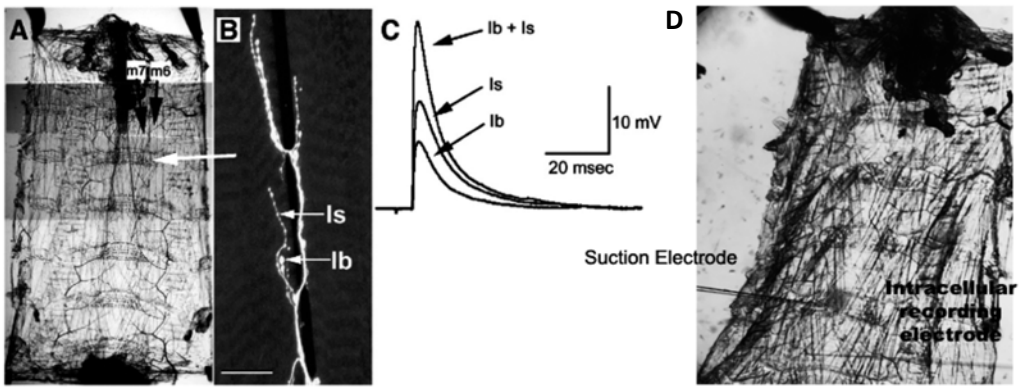

Figure 3.1 Semi-intact dissection.

A) Dissected larval Drosophila with muscles 6 and 7 labeled. B) Imaging of Is and Ib terminal boutons. C) Example of recruitment of both Is and Ib. D) Dissected preparation with the suction electrode and the intracellular recording electrode labeled

3.3 5Hz Continues Depression Experimental Protocol

Preparations were dissected via the semi-intact protocol, as described above. All preparations were dissected at the 3rd instar stage. An intracellular electrode was used to confirm a normal RP with the preparation. A suction electrode was used to provide electrical stimulation to the appropriate segmental nerve. Half Hz stimulation rate was used to determine an adequate voltage for producing a maximum response. The 5 Hz stimulation rate was then used to continuously produce EJPs in the preparation. Initial amplitude is determined off the first full EJP after the start of the 5 Hz stimulation. The stimulation continues until the average amplitude of the EJPs are half of the initial amplitude. The time from the start of the 5Hz stimulation until the point of 50% depression is recorded.

The control group was fed a standard, cornmeal-based diet for 24 hours. Each group was given a total of 5 grams of food. The 20% group was reared initially on the standard cornmeal-based diet, and then given 4 grams of the standard, cornmeal-based diet and 1 gram of coconut oil for 24 hours prior to recordings. The 40% group was reared initially on the standard cornmeal-based diet, and then given 3 grams of the standard, cornmeal-based diet and 2 grams of coconut oil for 24 hours prior to recordings. The two treatments groups were switched to their treatment diets at the 2nd instar stage.

Control preparations are maintained during stimulation in the modified HL3 saline. Preparations for the 20% fat-diet group and the 40% fat-diet group are maintained during stimulation in the modified HL3 saline. Those preparations are housed with the appropriate modified diet for 24 hours prior to dissection. Preparations with 10mM LiAA

are housed on the standard cornmeal diet. They are maintained during stimulation with a 10mM LiAA solution in the modified HL3 saline (Figure 3.2).

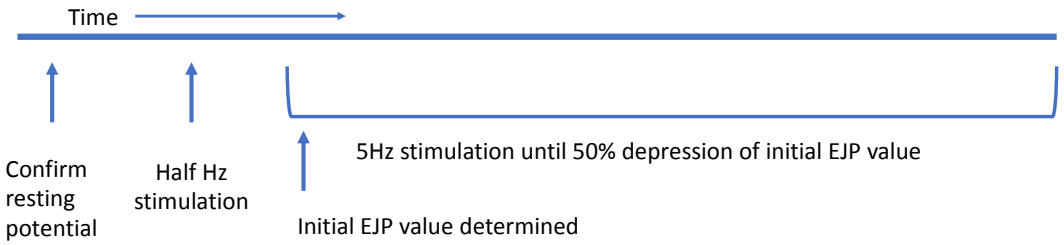

Figure 3.2 Experimental setup used to determine time to 50% depression of initial EJP amplitude.

An intracellular recording electrode confirms an acceptable RP. A suction electrode is used to deliver electrical stimulation to the appropriate segmental nerve. Half Hz stimulation is used to determine the voltage required to elicit a maximum response in order to ensure recruitment of both the Is and the Ib nerve terminal. 5Hz stimulation is used in order to elicit a fast rate of vesicular turnover. The 5Hz stimulation is continued until the point of 50% depression of the initial EJP amplitude.

3.4 Voltage Clamping - Quantal Events Protocol

Preparations are dissected via the semi-intact protocol, as described above. All preparations are dissected at the 3rd instar stage. An intracellular electrode is used to confirm a normal RP with the preparation. A suction electrode is used to provide electrical stimulation to the appropriate segmental nerve. A half Hz stimulation rate is used to determine an adequate voltage for producing a maximum response. The preparation is then voltage clamped using a single electrode with Axoclamp 2B. Voltage is manually maintained by injecting current during all periods in which it is clamped at -60mV (±3 mV). All stimulation is turned off and spontaneous quantal events are collected for a period of two minutes. The intracellular electrode is then removed from the preparation. 5Hz stimulation is delivered to the preparation through the suction electrode for a period of either 30 or 45 minutes.

Following this period, all stimulation is stopped. An intracellular recording electrode is used in the same muscle fiber used initially. Brief half Hz stimulation is used to ensure that the segmental nerve is still inside the suction electrode and has been delivering the 5Hz stimulation for the duration of the run. All stimulation is turned off and spontaneous quantal events are collected for a period of two minutes (Figure 3.3).

Spontaneous quantal events are analyzed from 30 second windows within both of the two-minute time periods for each run (Figure 3.4). Both frequency and amplitude of each event are recorded. Only events that are above the noise level of the preparation are able to be counted. Preparations in which the noise level is higher than normal are thrown out.

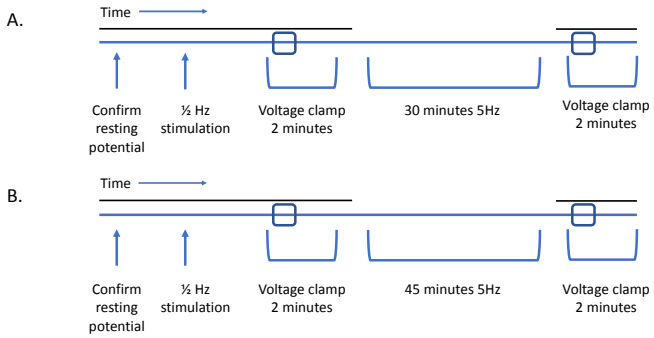

Figure 3.3 Experimental setup used to measure spontaneous quantal events in an evoked stimulation setting.

Black bars denote times in which an intracellular recording electrode is used. An intracellular recording electrode is used to confirm an acceptable RP. A suction electrode is used to provide electrical stimulation to the appropriate segmental nerve. Half Hz stimulation is used to determine the appropriate voltage required for a maximal response. Stimulation is turned off and the preparation is voltage clamped at 60mV (±3 mV). Spontaneous quantal events are collected for a period of two minutes at a high gain of resolution. The intracellular recording electrode is removed from the muscle during the designated period of 5Hz stimulation in order to ensure maximal integrity of the membrane; stimulation times are as indicated in the figure. Following the 5Hz stimulation, the intracellular recording electrode is reintroduced into the same muscle in the same spot as before. Half Hz stimulation is used briefly to ensure that the segmental nerve remained in the suction electrode for the duration of the stimulation period. The preparation is voltage clamped at 60mV (±3 mV) and spontaneous quantal events are collected for a period of two minutes at a high gain of resolution.

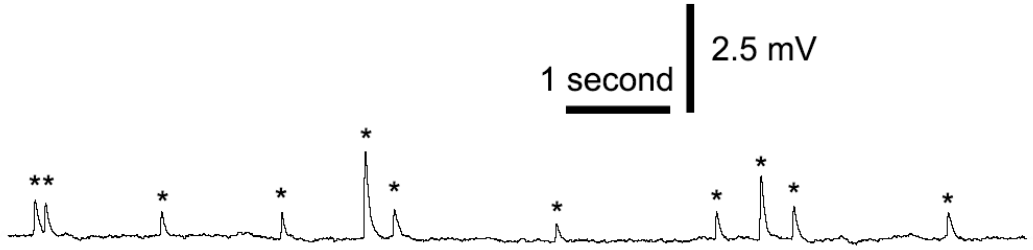

Figure 3.4 Spontaneous quantal event determination example.

A representative trace of non-evoked quantal events is shown. Each quantal event is marked by an asterisk. Quantal events are determined by the presence of the characteristic shape of the event: a fast, sharp rise followed by a slower degradation of the signal.

3.5 Half Hertz Amplitude Determinations Protocol

Preparations are dissected via the semi-intact protocol, as described above. All preparations are dissected at the 3rd instar stage. An intracellular electrode is used to confirm a normal RP with the preparation. A suction electrode is used to provide electrical stimulation to the appropriate segmental nerve. An agar bridge composed of 1% agar and modified HL3 solution is used in order to ensure that the ground wire remains in solution during bath changes. Modified HL3 saline is used in the first two minutes to establish a RP for the preparation. Half Hz stimulation is used throughout the preparation. A bath change to either LiAA or to saline is done and half Hz electrical stimulation is delivered for a period of 3 minutes. A final bath change to saline is done and half Hz electrical stimulation is delivered for a period of two minutes.

The RP is recorded throughout the preparation. The lowest RP value observed in each section (saline – treatment – saline) is used for statistical analysis. An average of three EJP amplitudes taken from the same location in each section of the preparation is also used for statistical analysis. Changes in RP changes in average EJP amplitude, and washout ability of LiAA are determined from this experimental protocol (Figure 3.5).

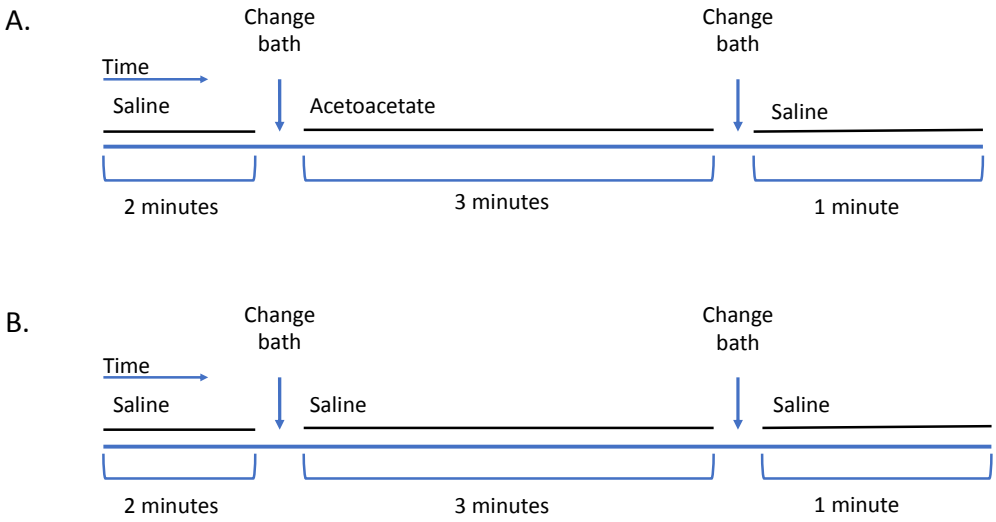

Figure 3.5 Experimental setup used to measure changes in RP and in EJP amplitude. An intracellular recording electrode is used to confirm an acceptable RP. A suction electrode is used to provide electrical stimulation to the appropriate segmental nerve. Half Hz stimulation is used to allow for a determination of the EJP amplitudes. This lower rate of frequency will prevent the stimulation from having any effect on resting membrane potential. An agar bridge housing the ground wire is used to minimize the effects of a bath change. RPs are recorded throughout. Average EJP amplitude is determined for each section of preparation.

3.6 Statistical analysis

Various statistical measures were employed; many data sets featured non-normal distributions of data and nonparametric statistical test were utilized. A detailed explanation of each statistical measure used is presented in conjunction with the results from the analysis in Chapter Four.

CHAPTER 4. RESULTS

The following results section is organized into three main categories: synaptic depression data, analysis on resting membrane changes, and spontaneous quantal events data. Within each category, multiple experiments were run in order to address each aim presented earlier. This chapter concludes with a concise summary of all pertinent results.

4.1 Synaptic depression

Synaptic depression studies are useful for characterizing rundown of EPSP amplitude over time. Efficient vesicle turnover is essential for maintaining the amplitude of EPSPs over time; if vesicle repackaging is being interfered with, the rundown of the amplitudes would decrease over time. A quicker run-down time could indicate that vesicle repackaging is being interfered with. For the following studies, time to 50% synaptic depression was determined. A suction electrode provided electrical stimulation to the segmental nerve innervating muscle 6 and 7. An intracellular recording electrode in muscle 6 or 7 recorded response to the provided stimulation. Initial amplitude was determined by the first observed full amplitude EJP at the start of 5Hz stimulation. Then, the time until the amplitudes averaged half of the amplitude was determined. A high stimulation rate (5Hz) was used in order to recruit vesicles from all pools (Figure 4.1).

4.1.1 Statistical Analysis

The data set failed the Shapiro-Wilk normality test ($P < 0.05$), so nonparametric statistical analysis had to be used. The Kruskal-Wallis One Way Analysis of Variance on Ranks assumes samples are not linked and independent. It can be used when comparing at least 3 groups on a dependent variable. The Kruskal-Wallis One Way Analysis of Variance on Ranks test produced a p value of 0.002, indicating significance within the dataset. To serve as a post-hoc analysis, multiple comparisons versus the control group were performed (via Dunn's method). All comparisons against control (saline) showed significance (20% p=0.023, 40% p=0.032, LiAA p= <0.001) (Figure 4.2).

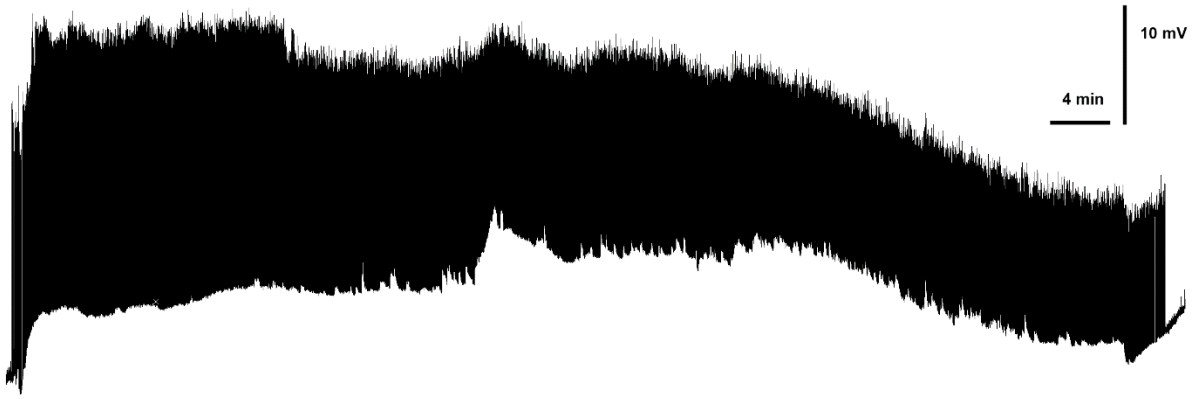

Figure 4.1: Representative trace for determination of time to 50% depression of EJP amplitude.

Initial EJP amplitude is determined off the first full EJP observed. The amplitudes of the EJPs are monitored until they reach an average value that is 50% of initial. RP is monitored throughout to ensure quality of the preparation.

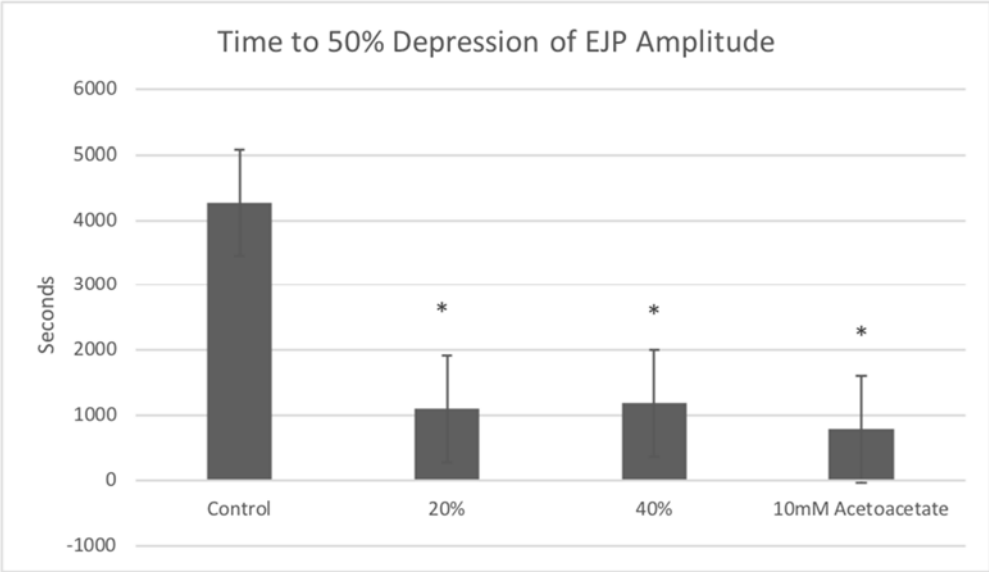

Figure 4.2: Time (in seconds) to a reduction in EJP amplitude by 50% with 5Hz stimulation.

Control flies were fed a standard cornmeal diet and dissected in modified HL3 saline. 20% and 40% treatment group were raised in modified diets containing higher levels of fat compared to the standard diet. 10mM acetoacetate treatment group was fed a normal cornmeal diet and then had 10mL LiAA in modified HL3 saline applied onto the dissected preparation. Stimulation is provided via a suction electrode; EJP responses are determined via an intracellular recording electrode. Initial amplitude is determined off the first full EJP at the start of the 5Hz stimulation. Data is presented as mean ± SEM. Statistical significance determined by a Kruskal-Wallis One Way Analysis of Variance on Ranks and Dunn's method post-hoc multiple comparisons versus the control ($n = 6$ for saline, 20%, and 40%, $n = 7$ for 10mM LiAA, $p < 0.05$ is considered to be statistically significant and is indicated by an asterisk).

4.2 Resting Membrane Potential Determination

Determining a compound's effect on the RP of the post-synaptic muscle may help to characterize possible post-synaptic effects. Understanding any post-synaptic effects is important for determining if other effect seen with other assays are being derived solely from a presynaptic effect. An intracellular recording electrode was used to determine membrane potential changes, if any, upon application of LiAA compared to modified HL3 saline. Additionally, after application of the appropriate treatment, modified HL3 saline was applied back onto the preparation to determine the potential for LiAA to be washed out (Figure 4.3).

4.2.1 Statistical analysis

For the resting membrane potentials recorded, a percent change from the initial RP of the saline bath to the treatment (LiAA or modified HL3) was calculated. Each preparation shows variation in the initial RP recorded, so a percent change allows comparison across preparation. A Shapiro-Wilk normality test was applied in order to verify a normal distribution of the data ($p=0.252$, pass). A Brown-Forsythe statistical test was used to verify equal variance within the data ($p=0.769$, pass). Because the data set has a normal distribution and equal variance, a 2-tailed t-test assuming equal variances was used to determine statistical significance. Application of LiAA causes a significant hyperpolarization of the resting membrane potential ($p=0.0000107$) (Figure 4.4).

The resting membrane potential recorded during the washout showed no statistical significance compared between the LiAA treatment group and the modified HL3 group, but did show a large standard error. It appeared that the ability of LiAA to washout was

inconsistent. Additionally, the washout affect was observed after 2 bath changes; it was consistently noted across many different experimental protocols that resting membrane potentials would often worsen after multiple bath changes. It is likely that the great degree of variance across preparations is due to this effect. Average amplitudes within each period of the preparation were also analyzed and showed no statistical significance (data not shown). The K(ATP) channel located on muscle membrane was ruled out as a possible target for LiAA that could cause this hyperpolarization. The use of Pinacidil, an agonist for the channel, did not produce a hyperpolarization of the RP (data not shown).

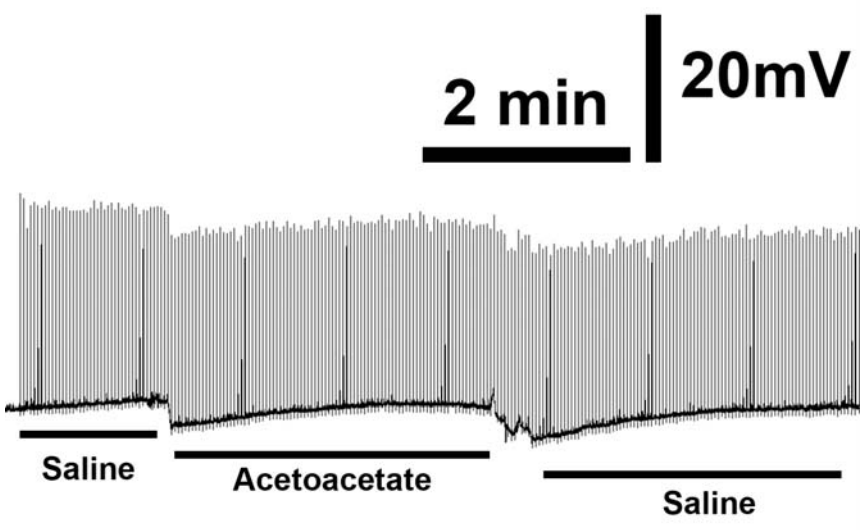

Figure 4.3 Representative trace for RP.

Black labeled bars indicated the solution that was on the preparation at that time. A noticeable hyperpolarization is evident upon application of 10mM LiAA. There is a slight change in the resting membrane potential with the saline washout; this was not consistent across all preparations.

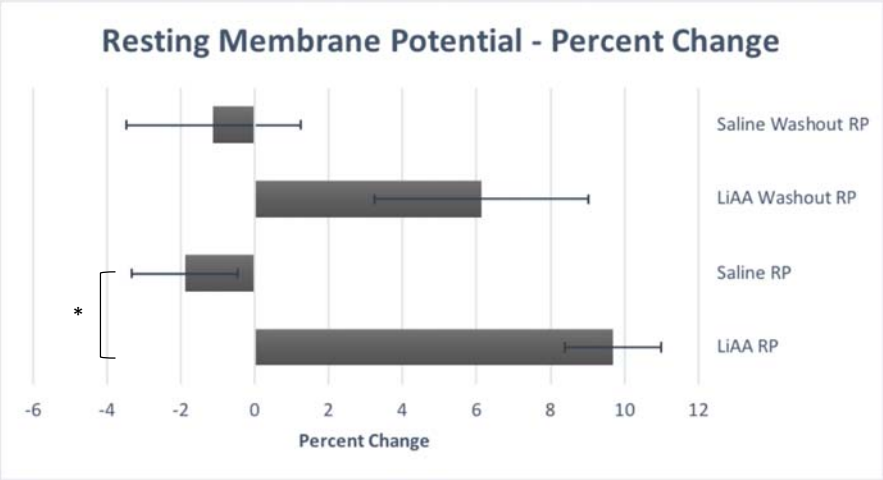

Figure 4.4 Percent change from original baseline RP value.

An intracellular recording electrode was used to determine the RP of each preparation; a glass suction electrode was used to provide half Hz electrical stimulation. Percent change values were calculated against the RP value that was determined at the start of the preparation. The treatment groups were either 10mM LiAA or modified HL3 (saline). Washout effect on the RP was also quantified. Data is presented as average percent change ± SEM. A negative percent change value is indicative of a depolarization relative to the original RP for the preparation. A positive percent change value is indicative of a hyperpolarization relative to the original RP for the preparation. (n=10 for each treatment group, 2-tailed equal variance t-test, $p<0.05$ is considered to be statistically significant and is indicated by an asterisk).

4.3 Spontaneous Quantal Events

Spontaneous quantal events are small post-synaptic events that occur without electrical stimulation. They can be useful for assaying both pre- and post-synaptic effects of a compound. A decrease in relative amplitude could be indicative of a decrease in efficient vesicle packaging, apart from any postsynaptic effects; it is possible that some postsynaptic effects could alter quantal events as well. A decrease in relative frequency can be indicative of a decrease in relative amplitude, and thereby a decrease in vesicle packaging as well. Smaller amplitude minis can be "lost" in the intrinsic noise level of a preparation, which could reflect as a decrease in relative frequency rather than relative amplitude. Analysis of quantal events must be multi-faceted in order to derive all effects and in order to explore the potential for effects seen to be resultant of pre or post synaptic effects (or both) (Figure 4.5, Figure 4.6).

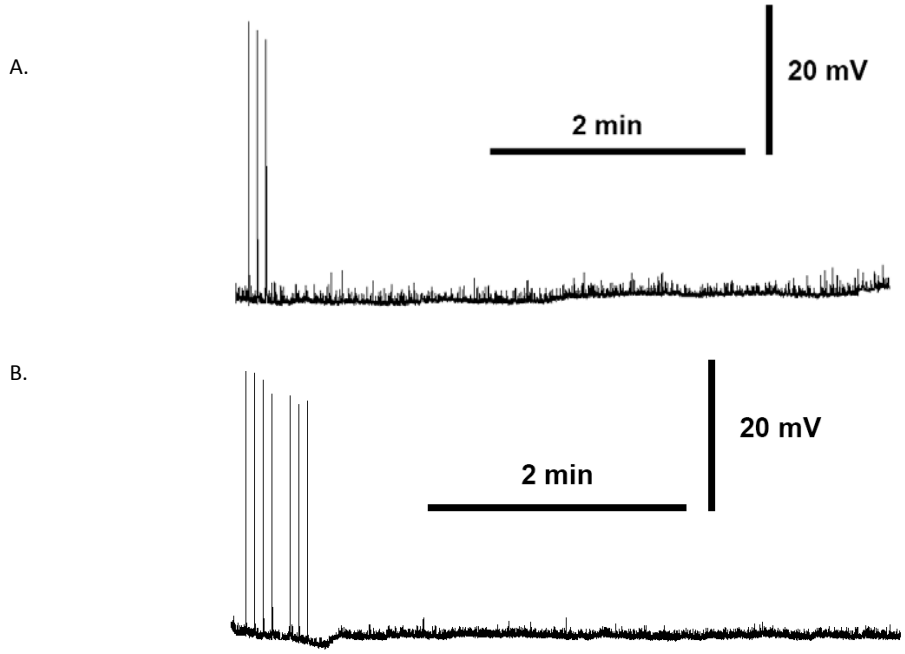

Figure 4.5 Representative trace for LiAA quantal events.

A) Recording taken prior to 5Hz stimulation. An intracellular recording electrode is used to record the quantal events and to inject current in order to maintain a RP of 60 mV (±3 mV). Initial responses are from half Hz stimulation, which is used to ensure that the glass suction electrode has the correct segmental nerve in it and the voltage being delivered is sufficient enough to derive a maximal response. Smaller responses following the initial EJPs are quantal events. B). Recording taken post 5Hz stimulation. Again, the intracellular recording electrode is used to record the quantal events and maintain the RP and a glass suction electrode is used to generate the initial EJP events in order to ensure that the segmental nerve was stimulated throughout the 5hz electrical stimulation period. Variation in quantal size and frequency can be seen when compared to A.

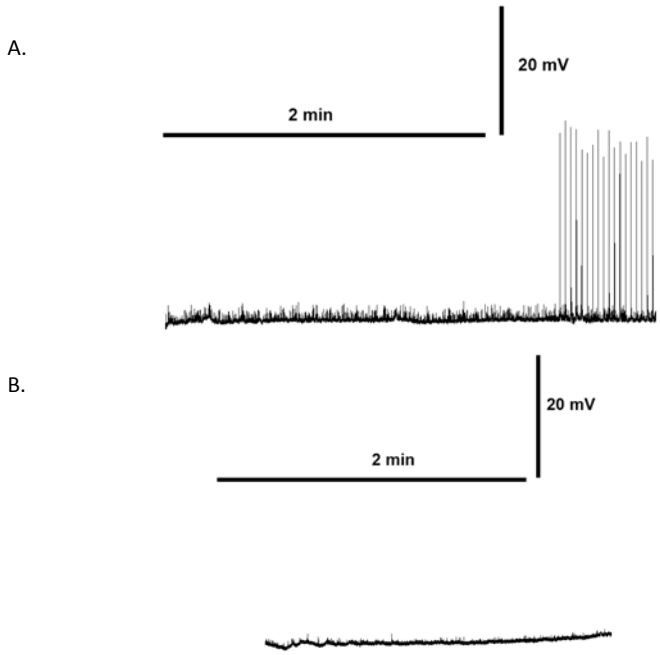

Figure 4.6 Representative trace for saline quantal events.

A) Recording taken prior to 5Hz stimulation. An intracellular recording electrode is used to record the quantal events and to inject current in order to maintain a RP of 60 mV (±3 mV). Responses towards the end of the trace are from half Hz stimulation, which is used to ensure that the glass suction electrode has the correct segmental nerve in it and the voltage being delivered is sufficient enough to derive a maximal response. Smaller responses prior to the EJPs are quantal events. B). Recording taken post 5Hz stimulation. Again, the intracellular recording electrode is used to record the quantal events and maintain the RP and a glass suction electrode is used to generate the initial EJP events (not shown) in order to ensure that the segmental nerve was stimulated throughout the 5hz electrical stimulation period. Variation in quantal size and frequency can be seen when compared to A.

4.3.1 Frequency of quantal events

Frequency of quantal events can show relative effects on efficient vesicular packaging. If vesicular packaging events are being interfered with and vesicular glutamate content is lower, then there may be an increase in the number of small quantal events and a decrease in the number of larger quantal events. Very small quantal events can be lost in the noise level, which would reflect as a lower frequency.

Frequency of quantal events were compared within treatment groups to assess rundown effects. Counts taken from a 30 second portion of the two minutes of quantal events recorded prior to 5Hz stimulation are compared against counts taken from a 30 second portion of the two minutes of quantal events recorded after the 5Hz stimulation. The two treatment groups were 10mM LiAA and modified HL3 (saline). Two different period of 5Hz stimulation were utilized (30 minutes and 45 minutes). 45 minutes of 5Hz stimulation will cause more vesicular turnover than the 30 minutes of 5Hz stimulation.

For the 10mM LiAA group with 45 minutes of 5Hz stimulation, a Shapiro-Wilk statistical test was used to verify a normal distribution of data ($p=0.118$, pass). A Brown-Forsythe statistical test was used to ensure equal variance ($p=0.102$, pass). A two-tail t-test with equal variance was used to determine statistical significance. There was a low statistical significance for the 10mM LiAA treatment group given 45 minutes of 5Hz stimulation ($p=0.0699$). No other treatment groups showed statistical significance (Figure 4.7).

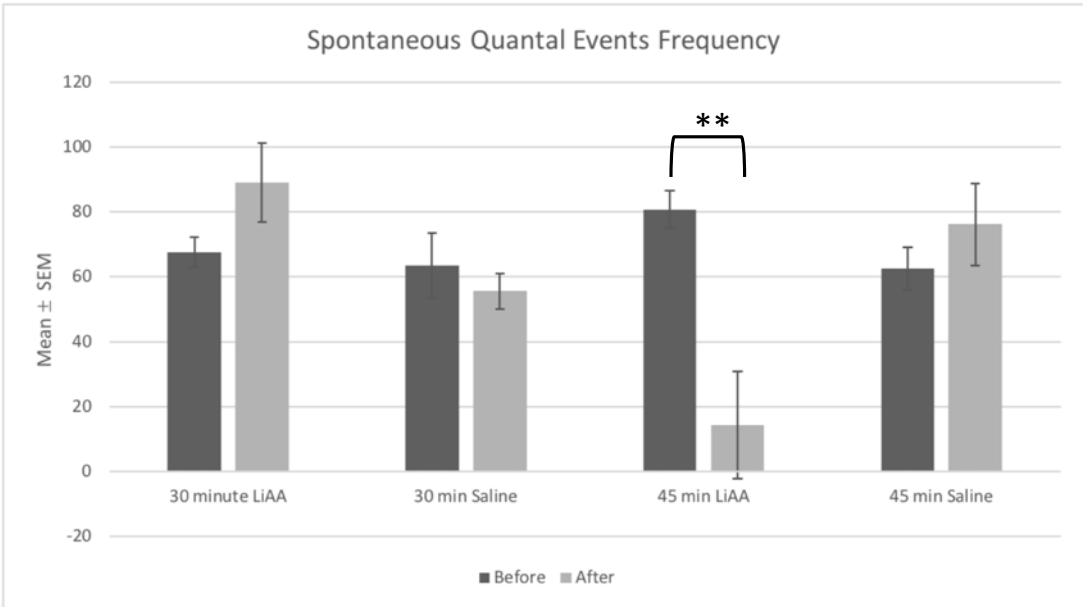

Figure 4.7 Quantal events frequency within treatment groups.

Quantal counts were taken from a 30 second portion of the two minutes of quantal events collected prior to 5Hz stimulation ("Before" group) and after 5Hz stimulation ("After" group). An intracellular recording electrode was used to record the quantal events. All preparations were voltage-clamped at 60mV (±3mV). Data is presented is mean ± SEM. Statistical significance was determined by a 2-tail t-test with equal variance (n=10 for all 4 treatment groups, $p<0.1$ is considered to be low-significant and is indicated by two asterisks).

All preparations were voltage clamped at 60mV (±3mv) and quantal counts were taken at the same time points within each preparation, which allows comparison across treatment groups. Comparisons were made at the time period prior to 5Hz stimulation and the time period after 5Hz stimulation. 10mM LiAA was compared against modified HL3 (saline) for both the 30 min 5Hz experimental protocol and the 45 min 5Hz experimental protocol.

For comparison of LiAA against saline prior to 5Hz stimulation for 45 minutes, a Shapiro-Wilk statistical test was used to ensure a normal distribution of the data ($p=0.465$, pass). A Brown-Forsythe statistical test was used to ensure equal variance ($p=0.577$, pass). Because the data featured a normal distribution and equal variance, a two-tailed t-test with equal variance was used to determine statistical significance. 10mM LiAA showed a higher average frequency of quantal events prior to 5Hz stimulation compared to saline. This had low-significance ($p=0.0512$).

For comparison of LiAA against saline after 5Hz stimulation for 30 minutes, a Shapiro-Wilk statistical test was used to ensure a normal distribution of the data ($p=0.206$, pass). A Brown-Forsythe statistical test was used to ensure equal variance ($p=0.16$, pass). A two-tailed t-test with equal variance was used to determine statistical significance. 10mM LiAA showed a higher average frequency of quantal events after 30 minutes of 5Hz stimulation compared to saline ($p=0.0207$).

For comparison of LiAA against saline after 5Hz stimulation for 45 minutes, a Shapiro-Wilk statistical test was used to check for a normal distribution of the data ($p<0.05$). Because the data did not feature a normal distribution, a Mann-Whitney statistical test with Yates continuity correction was used to determine statistical

significance. The Mann-Whitney statistical test assumes independent samples, a non-continuous dependent variable, and data that is not normally distributed (127). 10mM LiAA showed a higher average frequency of quantal events after 45 minutes of 5Hz stimulation compared to saline ($p=0.037$). There was no statistical significance for comparison of 10mM LiAA against saline prior to 5Hz electrical stimulation for 30 minutes (Figure 4.8).

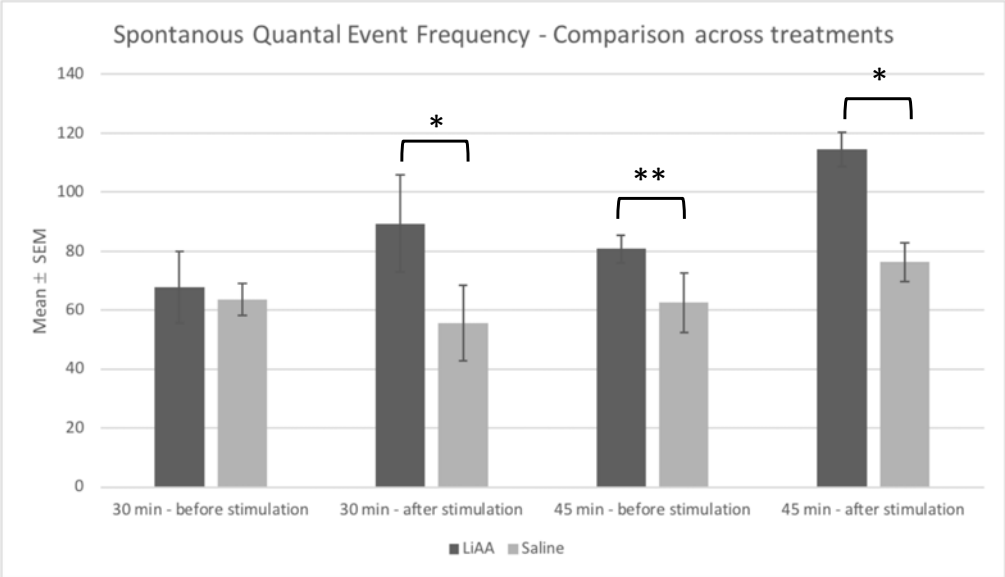

Figure 4.8 Quantal events frequency compared across treatment groups.

Quantal counts were taken from a 30 second portion of the two minutes of quantal events collected prior to 5Hz stimulation and after 5Hz stimulation. An intracellular recording electrode was used to record the quantal events. All preparations were voltage-clamped at 60mV (±3mV). Data is presented is mean ± SEM. Statistical significance for LiAA against saline frequency for prior to 45 minutes of 5Hz and frequency after 30 minutes of 5Hz was determined by a two-tail t-test with equal variances. Statistical significance for LiAA against saline for frequency after 45 minutes of 5Hz electrical significance was determined by a Mann-Whitney statistical test. N=10 for all treatment groups, $p < 0.05$ is statistically significant and is indicated by an asterisk, $p < 0.1$ is low-significant and is indicated by two asterisks.

4.3.2 Amplitude of Quantal Events

Amplitude of quantal events is another way to potentially infer a defect in vesicular glutamate packaging; smaller amplitude quantal events can be indicative of less glutamate content per vesicle. Again, changes in quantal events could also be a product of postsynaptic receptor blocking. It is important to note that a rundown of amplitudes post electrical stimulation is to be expected, even with saline controls. The frequency of amplitudes of quantal events are displayed below: data is presented as one representative histogram per group and a cumulative histogram per group. Amplitudes have been sorted into bins by relative size. All histograms trended towards a leftward shift in the relative frequency within amplitude bins after the 5Hz stimulation; amplitude size tended to decrease post electrical stimulation (Figure 4.9, Figure 4.10, Figure 4.11, Figure 4.12).

A Shapiro-Wilk test for normalcy was performed on quantal amplitudes for 10mM LiAA before and after 30 minutes of 5Hz stimulation. The data set did not have a normal distribution ($p < 0.05$), so a Mann-Whitney Rank Sum with Yates continuity correction was used to determine statistical significance. Quantal amplitudes were significantly lower after 5Hz stimulation ($p < 0.001$). The modified HL3 (saline) group for 30 minutes of 5Hz stimulation also failed the Shapiro-Wilk test ($p < 0.05$). The Mann-Whitney Rank Sum with Yates continuity correction was used to determine statistical significance. Quantal amplitudes were significantly lower after 5Hz stimulation ($p < 0.001$).

For the 10mM LiAA treatment group with 45 minutes of 5Hz stimulation, there was not a normal distribution of the data (Shapiro-Wilk, $p < 0.05$). The Mann-Whitney

Rank Sum with Yates continuity correction was used to determine statistical significance. Quantal amplitudes were significantly lower after 5Hz stimulation ($p < 0.001$). The modified HL3 (saline) group for 45 minutes of 5Hz stimulation also failed the Shapiro-Wilk test ($p < 0.05$). The Mann-Whitney Rank Sum with Yates continuity correction was used to determine statistical significance. Quantal amplitudes were significantly lower after 5Hz stimulation ($p < 0.001$).

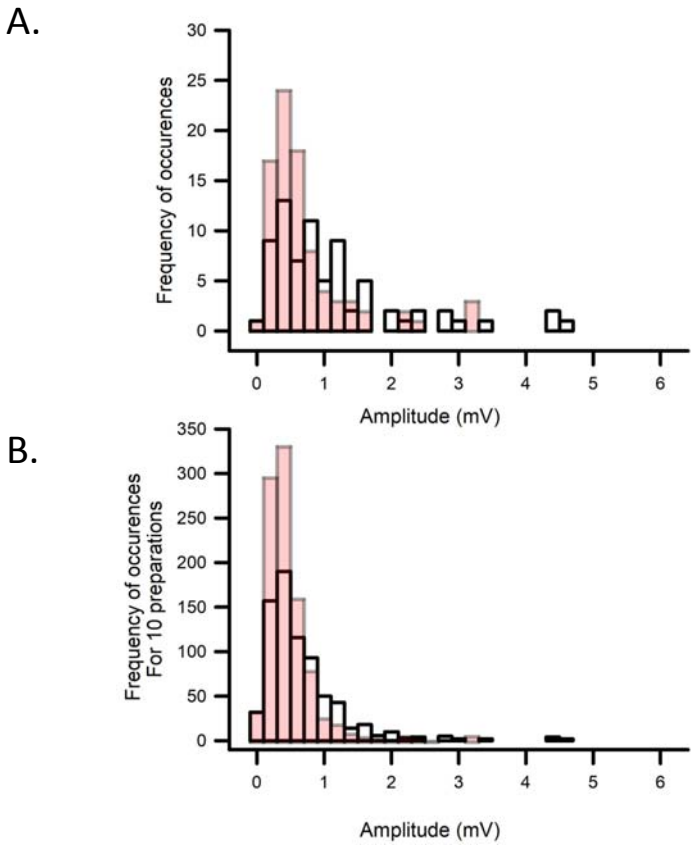

Figure 4.9 Histograms of frequency of different amplitude quantal events for 10mM LiAA before and after 30 minutes of 5Hz electrical stimulation.

Quantal amplitudes were taken from a 30 second portion of the two minutes of quantal events collected prior to 5Hz stimulation and after 5Hz stimulation for 30 minutes. An intracellular recording electrode was used to record the quantal events. All preparations were voltage-clamped at 60mV (±3mV). Clear bars are amplitudes taken prior to 5Hz. Red bars are amplitudes taken after 5Hz. A) A representative histogram taken from one preparation within the treatment group. B) Cumulative histogram from 10 preparations.

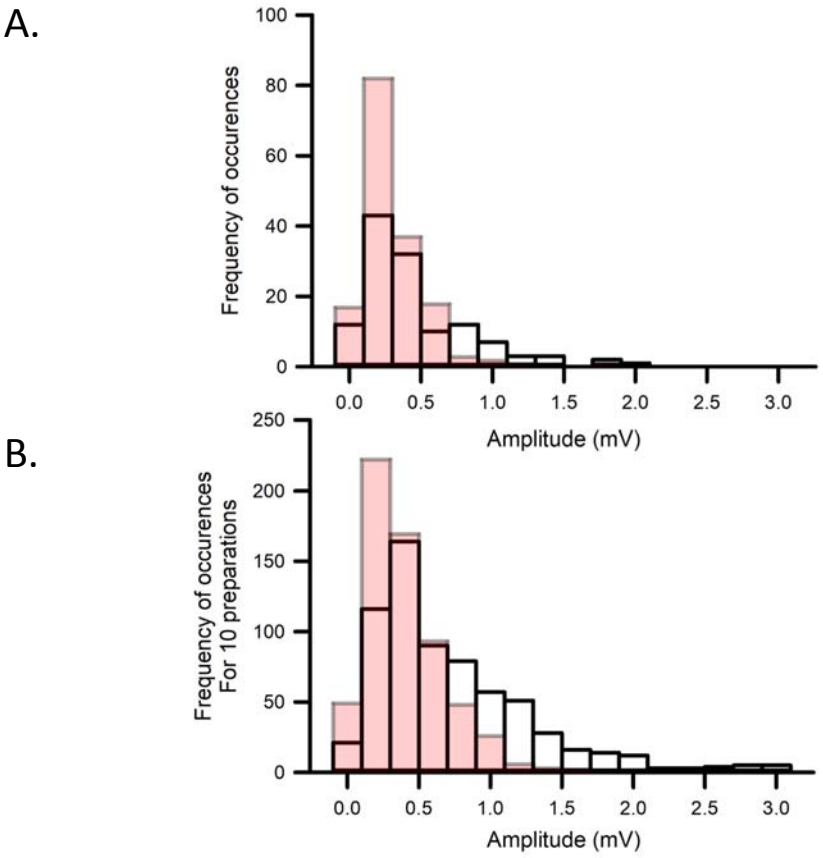

Figure 4.10 Histograms of frequency of different amplitude quantal events for modified HL3 before and after 30 minutes of 5Hz electrical stimulation.

Quantal amplitudes were taken from a 30 second portion of the two minutes of quantal events collected prior to 5Hz stimulation and after 5Hz stimulation for 30 minutes. An intracellular recording electrode was used to record the quantal events. All preparations were voltage-clamped at 60mV (±3mV). Clear bars are amplitudes taken prior to 5Hz. Red bars are amplitudes taken after 5Hz. A) A representative histogram taken from one preparation within the treatment group. B) Cumulative histogram from 10 preparations.

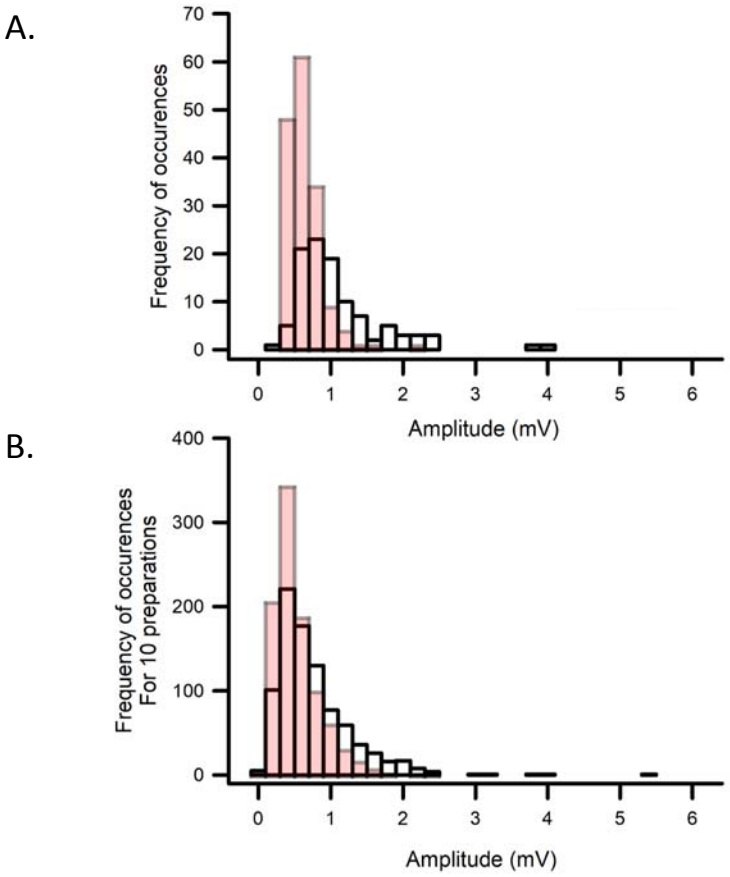

Figure 4.11 Histograms of frequency of different amplitude quantal events for 10mM LiAA before and after 45 minutes of 5Hz electrical stimulation.

Quantal amplitudes were taken from a 30 second portion of the two minutes of quantal events collected prior to 5Hz stimulation and after 5Hz stimulation for 45 minutes. An intracellular recording electrode was used to record the quantal events. All preparations were voltage-clamped at 60mV (±3mV). Clear bars are amplitudes taken prior to 5Hz. Red bars are amplitudes taken after 5Hz. A) A representative histogram taken from one preparation within the treatment group. B) Cumulative histogram from 10 preparations.

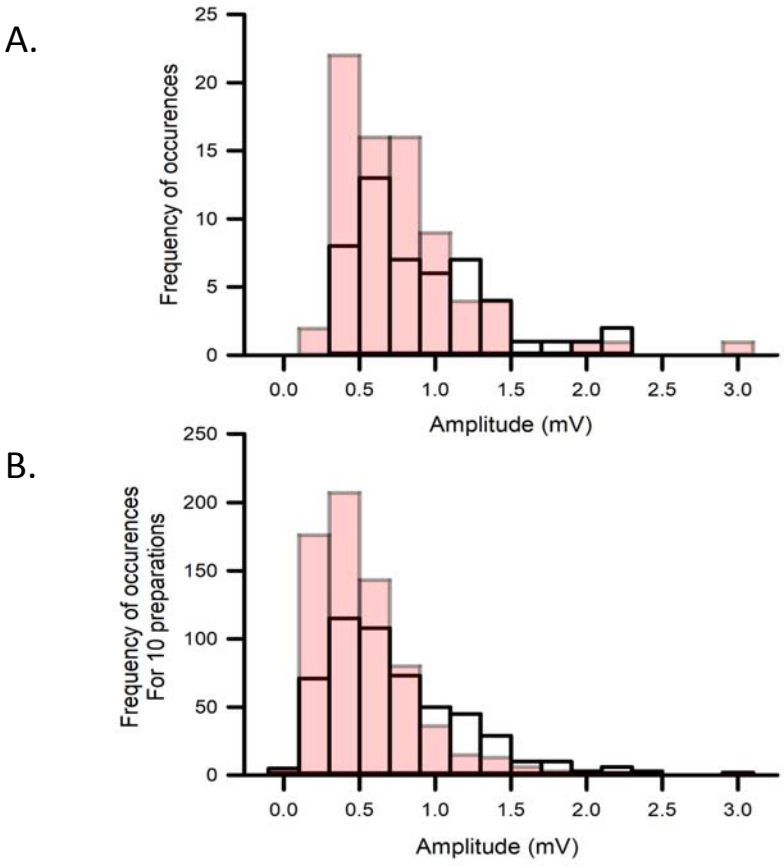

Figure 4.12 Histograms of frequency of different amplitude quantal events for modified HL3 before and after 45 minutes of 5Hz electrical stimulation.

Quantal amplitudes were taken from a 30 second portion of the two minutes of quantal events collected prior to 5Hz stimulation and after 5Hz stimulation for 45 minutes. An intracellular recording electrode was used to record the quantal events. All preparations were voltage-clamped at 60mV (±3mV). Clear bars are amplitudes taken prior to 5Hz. Red bars are amplitudes taken after 5Hz. A) A representative histogram taken from one preparation within the treatment group. B) Cumulative histogram from 10 preparations.

It was not unexpected that all treatment groups would show a statistically significant decrease in amplitude after electrical stimulation. To further investigate any difference in quantal event amplitudes between LiAA and modified HL3, the Kolmogorov-Smirnov test can be used to look at statistical differences between data sets. This test can be used when data sets are not normally distributed and may vary greatly in the number of data points between sets.

Comparing amplitudes for 10mM LiAA prior to 5Hz electrical stimulation and after 30 minutes of 5Hz electrical stimulation, the Kolmogorov-Smirnov test showed significance ($D = 0.20169$, $p < 0.0001$) (Figure 4.13). Comparing amplitudes for modified HL3 (saline) prior to 5Hz electrical stimulation and after 30 minutes of 5Hz electrical stimulation, the Kolmogorov-Smirnov test showed significance ($D = 0.2808$, $p < 0.001$) (Figure 4.14). Comparing amplitudes for 10mM LiAA prior to 5Hz electrical stimulation and after 45 minutes of 5Hz electrical stimulation, the Kolmogorov-Smirnov test showed significance ($D = 0.23048$, $p < 0.0001$) (Figure 4.15). Comparing amplitudes for modified HL3 (saline) prior to 5Hz electrical stimulation and after 45 minutes of 5Hz electrical stimulation, the Kolmogorov-Smirnov test showed significance ($D = 0.484$, $p < 0.001$) (Figure 4.16).

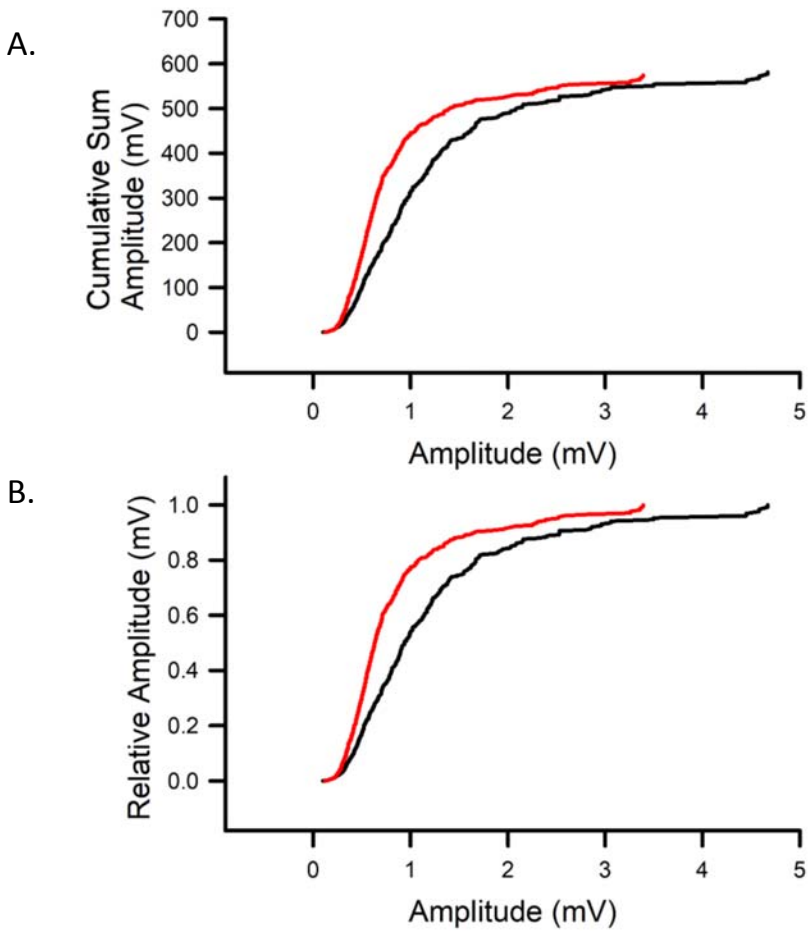

Figure 4.13 Kolmogorov-Smirnov plot of amplitude values for 10mM LiAA before and after 30 minutes 5Hz electrical stimulation.

Black lines are values prior to 5Hz. Red lines are values after 5Hz. A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Relative amplitude Kolmogorov-Smirnov plot; amplitude values normalized into the highest amplitude value within the set.

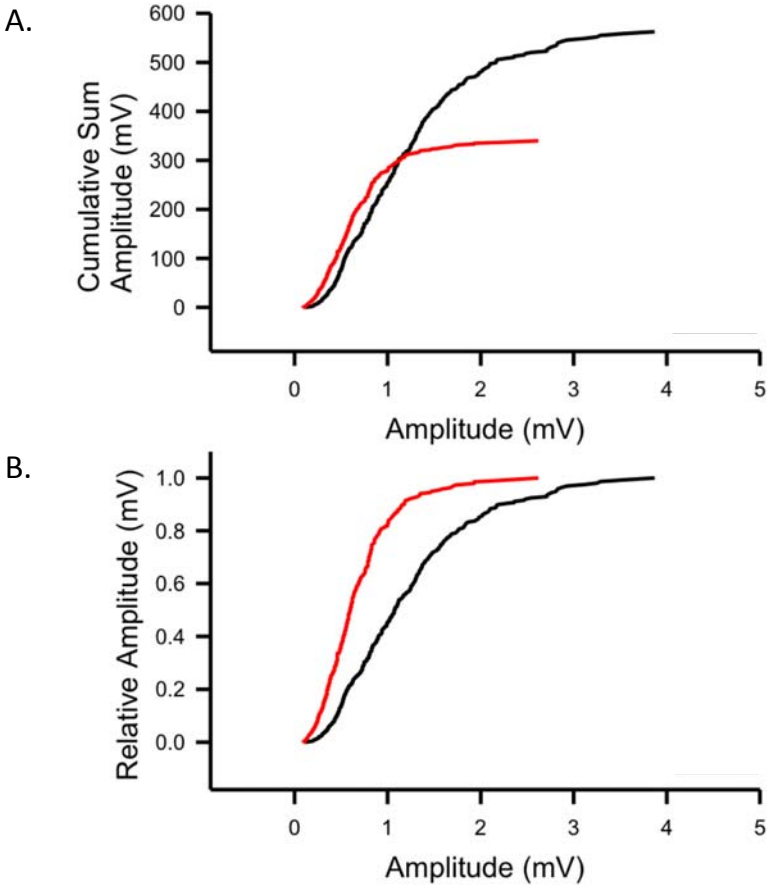

Figure 4.14 Kolmogorov-Smirnov plot of amplitude values for modified HL3 (saline) before and after 30 minutes 5Hz electrical stimulation.

Black lines are values prior to 5Hz. Red lines are values after 5Hz. A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Relative amplitude Kolmogorov-Smirnov plot; amplitude values normalized into the highest amplitude value within the set.

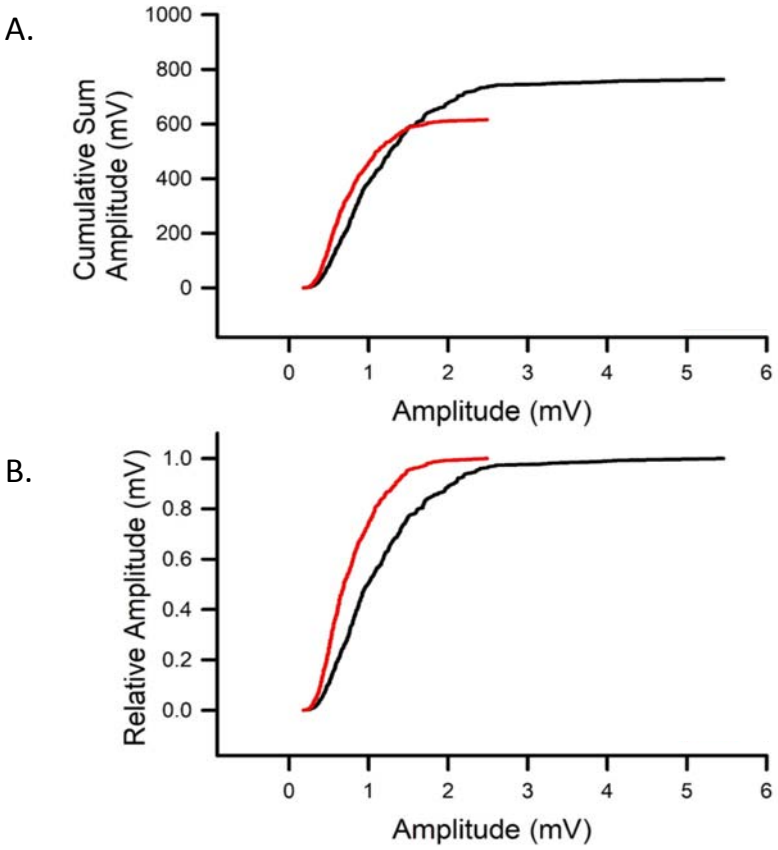

Figure 4.15 Kolmogorov-Smirnov plot of amplitude values for 10mM LiAA before and after 45 minutes 5Hz electrical stimulation.

Black lines are values prior to 5Hz. Red lines are values after 5Hz. A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Relative amplitude Kolmogorov-Smirnov plot; amplitude values normalized into the highest amplitude value within the set.

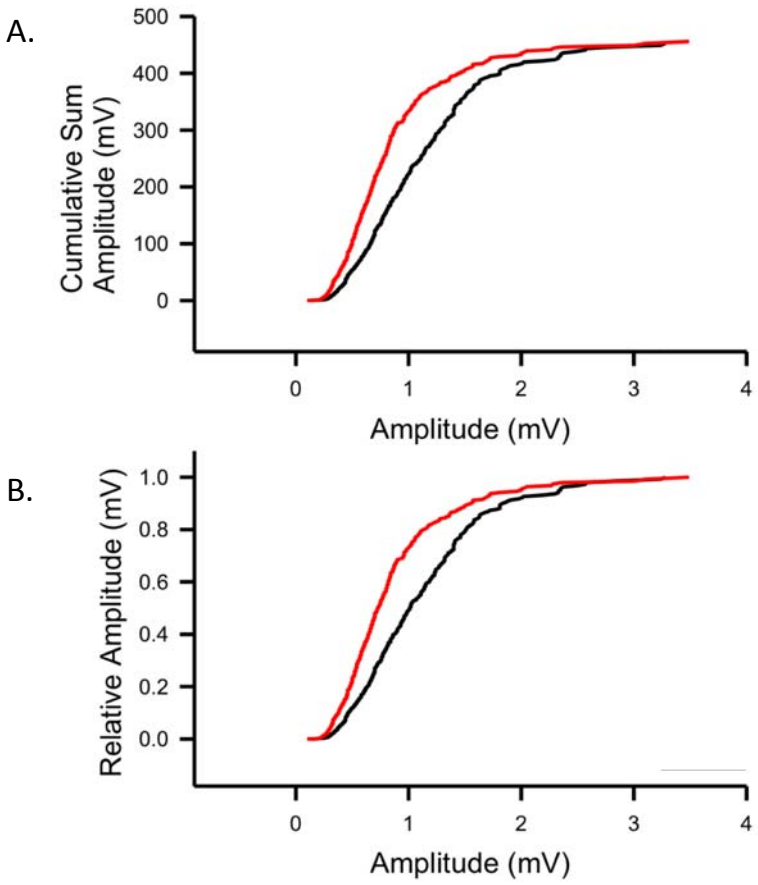

Figure 4.16 Kolmogorov-Smirnov plot of amplitude values for modified HL3 (saline) before and after 45 minutes 5Hz electrical stimulation.

Black lines are values prior to 5Hz. Red lines are values after 5Hz. A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Relative amplitude Kolmogorov-Smirnov plot; amplitude values normalized into the highest amplitude value within the set.

The following four figures (Figure 4.17, Figure 4.18, Figure 4.19, and Figure 4.20) display Kolmogorov-Smirnov plots of amplitude values between treatment groups. All amplitudes were taken from the same time period within the trace and all traces were voltage clamped at 60mV (±3mV). Comparisons of 10mM LiAA against modified HL3 are made prior to and after 5Hz stimulation (30 minutes and 45 minutes). All traces show no apparent difference between relative amplitudes between treatment groups.

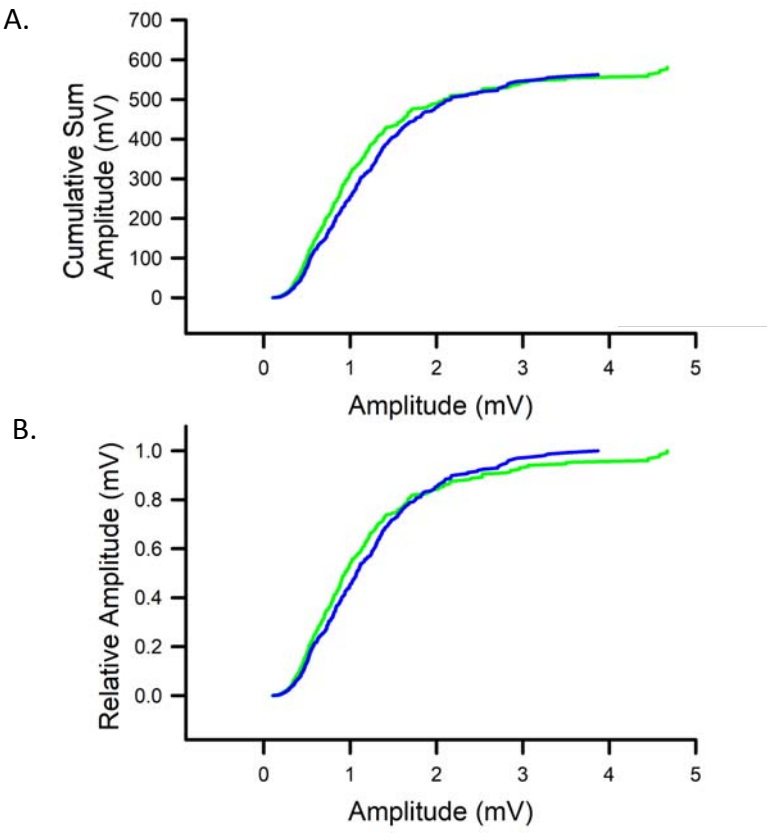

Figure 4.17 Kolmogorov-Smirnov plot of amplitude values prior to 30 minutes of 5Hz stimulation for 10mM LiAA versus modified HL3.

Green lines are 10mM LiAA. Blue lines are modified HL3 (saline). A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Amplitude values normalized into the highest amplitude value within the set.

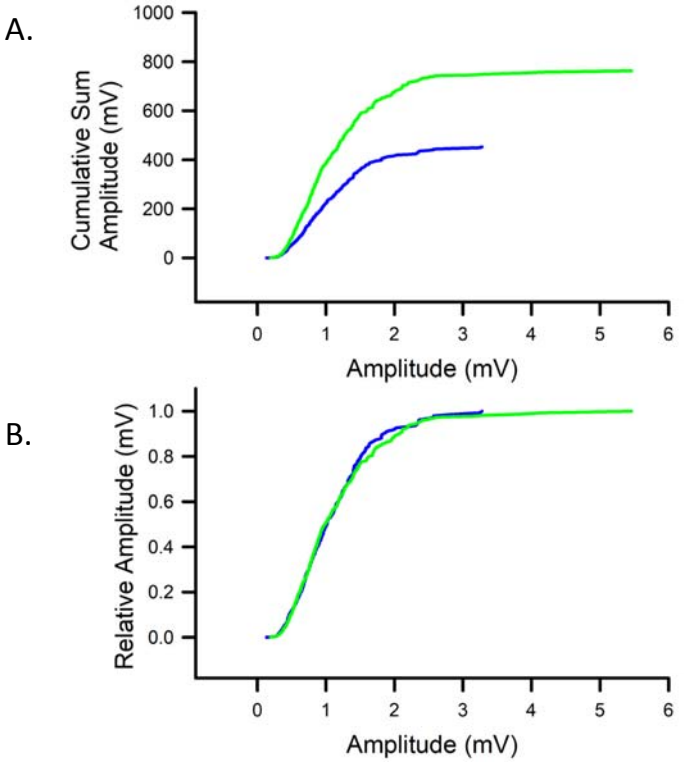

Figure 4.18 Kolmogorov-Smirnov plot of amplitude values prior to 45 minutes of 5Hz stimulation for 10mM LiAA versus modified HL3.

Green lines are 10mM LiAA. Blue lines are modified HL3 (saline). A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Amplitude values normalized into the highest amplitude value within the set.

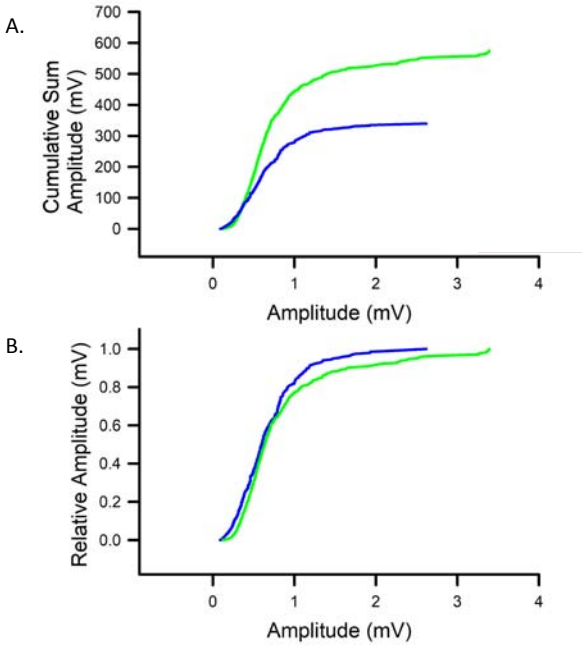

Figure 4.19 Kolmogorov-Smirnov plot of amplitude values after 30 minutes of 5Hz stimulation for 10mM LiAA versus modified HL3.

Green lines are 10mM LiAA. Blue lines are modified HL3 (saline). A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Amplitude values normalized into the highest amplitude value within the set.

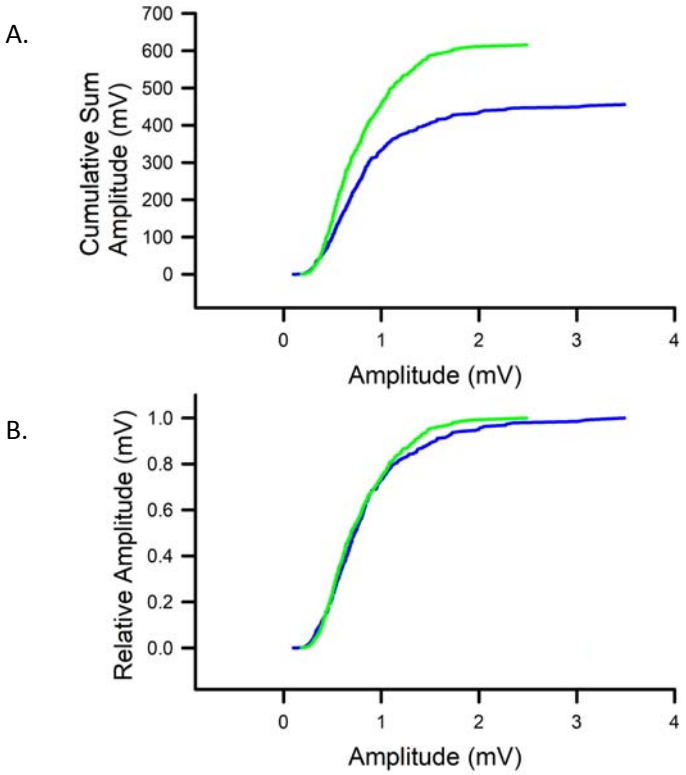

Figure 4.20 Kolmogorov-Smirnov plot of amplitude values after 45 minutes of 5Hz stimulation for 10mM LiAA versus modified HL3.

Green lines are 10mM LiAA. Blue lines are modified HL3 (saline). A) Cumulative sum Kolmogorov-Smirnov plot showing relative distribution of amplitudes. B) Amplitude values normalized into the highest amplitude value within the set.

4.4 Summary of Results

10mM Lithium Acetoacetate causes a significantly quicker rundown of EJPs compared to modified HL3 saline controls. This effect is replicated in both diets (20% fat content and 40% fat content). 10mM LiAA causes an immediate significant hyperpolarization of the muscle membrane upon application without causing a significant change in EJP amplitudes. The K(ATP) channel was ruled out as a possible target of LiAA that could cause this hyperpolarization. 10mM LiAA causes a significant increase in frequency of quantal events after both 30 minutes and 45 minutes of 5Hz electrical stimulation. Both saline and LiAA produce a rundown in amplitudes of quantal events for both 30 minutes and 45 minutes of stimulation. There is no difference in the relative rundown of these amplitudes across treatment groups.

CHAPTER 5. DISCUSSION AND FUTURE DIRECTIONS

5.1 Introduction

The results presented above are sufficient to address the specific aims of this thesis. They tend to be relatively in-line with the model of acetoacetate modulation proposed by Juge, but do point to other effects of acetoacetate beyond what was described previously. The findings in this thesis are important because they document several previously unknown effects of acetoacetate and are able to be integrated into the context of previous literature.

5.2 EJP amplitude reduction over time

EJPs are graded responses produced in the muscle at the *Drosophila* NMJ. The amplitude of a graded response can be indicative of the amount of neurotransmitter released into the synapse. Measuring synaptic depression, or relative rate of the decrease in EJP amplitude over time, is primarily a measure of presynaptic effects. However, there is the potential for postsynaptic effects to alter amplitude; potential postsynaptic effects must be considered when interpreting depression data.

The 5Hz electrical stimulation is used in depression studies in order to induce rapid vesicular turnover. Over time, this high rate of stimulation will cause vesicle recruitment from the reserve pools of vesicles. Based on the model proposed by Juge, blocking of glutamate packaging relies on two main factors: acetoacetate must bind the allosteric binding site on the VGLUT and the vesicle must first be emptied of already packaged glutamate. Using a high rate of stimulation should ensure that vesicles are being depleted

of glutamate in a rapid and widespread manner during the initial fusion event. Acetoacetate then would be able to block the subsequent refilling of these vesicles. Application of 10mM LiAA onto the preparation reduced EJP amplitude at a quicker rate when compared to saline preparations, which is in-line with the model.

Two different levels of high-fat diets were able to produce equivalent reductions in EJP amplitudes. Using diet to induce the same affects as direct application of LiAA onto the preparation serves two purposes: it validates the results seen with this experimental paradigm and it validates the results seen in subsequent experimental paradigms that rely solely upon application of LiAA onto the preparation.

While this assay can provide strong evidence for acetoacetate blocking vesicular turnover, it cannot stand alone. As mentioned above, the experimental paradigm fails to show that results are solely due to a presynaptic effect. Acetoacetate could be blocking receptors or certain ion channels on the postsynaptic membrane – this would produce an equivalent response.

There are certain limitations with this experimental paradigm. An intracellular recording electrode is used in the preparation throughout the duration of the 5Hz stimulation. For some preparations, this was as much as two hours. Having an electrode embedded within the muscle membrane for durations such as that can possibly compromise the membrane itself, which would alter the EJPs. One way to monitor the integrity of the membrane is to watch the RP and ensure that the value does not change too much.

All preparations did have some change in RP with this experimental paradigm; having the RP shift proved to be unavoidable. Any preparation that exhibited drastic changes in the RP was thrown out to try and prevent compromised muscle membrane integrity from skewing the data. Even with this caution in place, the change in RP could be driving some of the changes seen in EJP amplitude. Often, the RP would tend to depolarize relative to the initial RP over the duration of the preparation. This depolarization could decrease the driving gradient for ions; a decreased driving gradient could produce smaller EJPs. It is important to note that the changes seen with RP was seen for all treatment groups, including the saline controls. This should help to minimize the relative effect that this has on interpreting the results compared across treatment groups. Overall, this electrophysiological measure is commonly used to measure synaptic depression and is an accepted measure for this despite its limitations.

One alternative to depression studies performed like this one would be to voltage clamp the membrane. Voltage clamping involves injecting current through an intracellular recording electrode in order to maintain a specific membrane voltage. Maintaining the voltage at a specific value may allow better comparison across preparations and would prevent changes in the RP from altering the driving gradient, which could change EJP amplitudes. Voltage clamping with an experimental paradigm like this one could prove to be problematic for maintenance of the integrity of the membrane; injecting current for long durations of time can disrupt the membrane and cause changes to observed EJP amplitudes.

Voltage clamping was used in a different experimental paradigm. These results are discussed below. The voltage clamping experimental paradigm avoided the problem of

compromising the membrane integrity by only injecting current for a total of four minutes; electrical stimulation is done apart from the voltage clamping and just serves the purpose of eliciting high rates of vesicular turnover. The results from that experiment pair nicely with the results obtained from this experiment.

5.3 Hyperpolarization of the resting membrane

Application of LiAA onto a preparation immediately caused a hyperpolarization of the RP of the muscle. This hyperpolarization is likely evidence of a postsynaptic effect. The relative speed of the hyperpolarization (typically seen in under one-minute post application onto the preparation) rules out the hyperpolarization as a presynaptic effect. There are many mechanisms by which this hyperpolarization could be occurring. One could postulate that acetoacetate could be directly interfering with ion channels (i.e. potassium channels) to cause this hyperpolarization, or it could be indirectly offsetting flux of ions because the molecule itself is acidic and carries a formal charge; however, it has been shown that a lower pH will tend to depolarize larval *Drosophila* muscle (132). There is currently no evidence to support this notion, but could be a possible future avenue of research.

Interestingly, the hyperpolarization did not accompany a statistically significant change in EJP amplitude. This result was unexpected but not necessarily unexplainable. The hyperpolarization produced by acetoacetate was consistent and immediate but was not relatively large (the average change was just over 5mV). This small change may not be quite large enough to produce a consistent enough change in relative amplitude to be considered statistically significant. There was a great degree of variation on the relative

change of amplitude seen. The physical disturbance of changing the saline likely is one reason the RP showed some changes; it may have had a slight, but not significant, overall effect on the EJP amplitude.

Despite there being no evidence of a change in the relative EJP amplitude, the hyperpolarization could still be altering driving gradients for ion flux. This could alter amplitudes seen for both quantal events and EJPs in different assays and should be considered when interpreting data. An increase in the driving gradient likely is either not interfering significantly with the amplitudes, or it would keep the amplitudes increased for a longer relative period of time. If this effect was affecting amplitudes seen with the 5Hz depression studies, it would likely be in a manner that would increase the time to 50% depression rather than decreasing it, so the results for the time to 50% depression are still valid.

The biggest implication of the results of this assay is the evidence for acetoacetate acting in a postsynaptic manner. Prior to this, the literature supporting the model of acetoacetate binding the allosteric regulation site on a VGLUT has been entirely focused on presynaptic measures. This study points to a postsynaptic effect, but does not rule out the potential for depression results to be primarily caused a presynaptic effect. Instead, it introduces another variable: acetoacetate may be altering synaptic transmission in a multi-faceted way.

5.4 Increase in quantal frequency

Spontaneous quantal events are an ideal measure for exploring a compound's ability to alter synaptic transmission in both a pre and post synaptic fashion. These quantal events occur in a non-evoked manner. They are characterized by a significant degree of variation in both localized frequency and amplitude; they are relatively small in size.

A change in the frequency can derive from a number of mechanisms. An alteration in vesicular packaging of glutamate, as proposed by the Juge model, should decrease the relative amplitude of quantal events. Likely, this would manifest itself as a decrease in frequency of the quantal events. Very small quantal events are unable to be consistently counted because they are not significantly larger than the electrical noise level of the preparation. The number of events actually occurring may truly be the same, but it would manifest as a lower number due to the missing events. An additional mechanism by which quantal frequency could be decreased would be through the blocking of postsynaptic glutamatergic receptors. It was found that application of 10mM LiAA on the preparation caused an increase in the frequency of events seen. This is in direct conflict with the Juge model.

This increase in frequency could potentially point to a postsynaptic effect, via a feedback mechanism to the presynaptic terminal or change input resistance of the postsynaptic fiber, allowing for the very small quantal event to be above the noise level and now observable. However, the only postsynaptic effect that has been confirmed at this point is a hyperpolarization of the muscle membrane. All preparations for this assay

were voltage clamped at 60mV (±3mV), which effectively eliminates this hyperpolarization normally seen. This should also eliminate the potential for the driving gradient to be altered. An alteration in the driving gradient could cause an artificial inflation of quantal amplitudes, so this is an important control for these preparations.

Voltage-clamping is not a perfect way to prevent the effects of a postsynaptic from skewing data, especially because the mechanisms by which these postsynaptic effects are arising is not known. Membrane voltage is maintained in a voltage-clamped preparation by injecting current. This current can alter the relative membrane potential, but will not offset any changes in ion concentrations being caused by acetoacetate. Hyperpolarization of a membrane is often due to ion flux, which could change relative concentrations of that ion inside and outside the membrane. This change in ion concentration could still be affecting the quantal event responses being seen in the muscle.

Taken in the context of the limitations seen with voltage clamping, these results still point to the Juge model being at least incomplete, if not wrong. An increase in frequency could indicate that vesicle packaging is being maintained (or is possibly remaining more efficient compared to control). It has been proposed in prior literature that there is possibly a separate pool of vesicles that are reserved exclusively for these quantal events. The working model is that these vesicles are not used during evoked stimulation. The mechanism by which these vesicles are spared is not known. Evidence points to the effect not being a lack of response to calcium stimulation (a normal trigger for vesicular endocytosis into the synapse) because it has been shown that a increase in calcium can increase quantal events (133).

The increase in frequency of quantal events does not contradict this theory of vesicle sparing. Vesicles typically sit in the synaptic bulb pre-filled with neurotransmitter. As mentioned above, acetoacetate is thought to interfere with the repackaging of the glutamate. In order for there to be a repackaging event there would have to first be an endocytosis event. If there were to be a pool that is spared, then it is possible that acetoacetate would not be interfering with those vesicles during the 5Hz stimulation, leaving those vesicles intact.

This does not explain the relative increase from saline, but rather a lack of a decrease in frequency. Vesicles could be spared in this separate pool and there could be a relative increase in frequency due to changes in ion concentrations from ion channel opening or closing on the postsynaptic membrane. Changes in ion concentration could alter relative driving gradient for the glutamatergic ionotropic channels; ion flux post neurotransmitter binding to a receptor could be increased. This increase in ion flux could keep more quantal events above the level of noise. The relative increase seen may be entirely due to higher amplitudes rather than a true increase. Another possible mechanism by which quantal events could be enlarged would be the closing of potassium leak channels. This would increase the input resistance, which would increase the quantal size. Again, this data is in-line with a multifaceted action of acetoacetate. There are many mechanisms in which a change in frequency of quantal events could be indicative of a postsynaptic effect. These results appear to be less evident of a presynaptic mechanism of action for acetoacetate, at least in the context of quantal events.

5.5 Decrease in quantal amplitude after stimulation but no decrease in amplitude across treatment groups

Within each preparation, there was a statistically significant decrease in quantal amplitude. Amplitude measures were taken over 30 second periods both before and after 5Hz stimulation. This decrease was not unexpected, but serves as a primary indicator that the 5Hz stimulation is working to induce general vesicular turnover. General rundown with both saline and acetoacetate should be occurring due to a high rate of vesicle turnover.

Of more relevance is the lack of a difference in relative amplitudes across treatment groups. If acetoacetate was acting in the presynaptic fashion proposed by Juge, one would expect to see a relative decrease in amplitude when comparing the acetoacetate preparations against the saline preparations, especially post 5Hz stimulation or even prior to the stimulation, once LiAA is introduced to the preparation. Again, the lack of statistical significance here points to a possible postsynaptic mechanism by which acetoacetate is working. Acetoacetate could still be altering vesicular packaging of glutamate, but postsynaptic effects may be offsetting the alteration in a manner that produces results similar to saline controls.

5.6 Summary

Primarily, the data presented shows that the working model that has been proposed in previous literature is incomplete. The synaptic depression data still points to a likely interference in vesicular packaging. However, the work presented in this thesis provides

strong evidence of postsynaptic effects. The membrane hyperpolarization is direct evidence of postsynaptic mechanism of action that cannot be explained by the Juge model. The quantal evidence could suggest a possible postsynaptic mechanism and may also support, in part, the Juge model.

The work done by Juge is convincing on the validity of their findings. It is likely that acetoacetate is binding the proposed allosteric regulatory site and altering vesicular repackaging. They showed a chloride dependence of VGLUT repackaging and were able to replicate this general effect across many different assays. Taking this into consideration, it is likely that acetoacetate is acting a multi-faceted manner.

Research like this is of great importance because understanding the complete mechanism of action of acetoacetate may improve epilepsy drug development efforts. As stated previously, many patients are not responsive to the available medications. Patients that are responsive still have to deal with many unpleasant side effects. It has been well established prior to this that a ketogenic diet is an efficacious treatment. However, the literature supporting the mechanism by which this diet is acting to provide these effects is largely inconsistent and many different studies contradict each other. The proposed Juge model is attractive because of its simplicity and it is supported by several previous studies. The research in this thesis does not invalidate the model, but rather points to the model being simply incomplete.

5.7 Future Directions

The research conducted for this thesis has given rise to many more experimental questions that would need to be answered in order to determine a better working model of action. The *Drosophila* NMJ serves as an excellent model for studying the mechanism of action of acetoacetate and there are many more viable assays that could be done with *Drosophila* to further specify all manners by which acetoacetate is working.

The quantal data presented in this thesis is perhaps an area that could be explored first. The data sets produced for those assays are extremely large and there are many different, valid ways in which the data could be organized. Rigorous statistical analysis done in the context of several different organization schemes could possibly show effects that were not evident from the statistical analysis done for this thesis. More data could be pulled from the traces as performed for the quantal analysis. Quantal amplitudes and counts were only taken from two 30-second bins, but a total of 4 minutes of voltage-clamped non-evoked recordings were taken for each preparation. The data set could be enlarged to include longer time-periods of quantal analysis. It has been shown in previous literature that vesicles can still recycle while empty; imagining done with dyes like FM1-43 could be performed to verify that this phenomenon is happening in this model organism.

Paired-pulse ratios could also be performed on preparations to investigate if there is a change in relative probability of quantal release events. All of the data in this thesis assumed there to be no change in probability because it was shown prior that acetoacetate did not affect paired-pulse ratios. However, the work was not done on this particular

model; doing paired-pulse ratios could verify that all quantal effects seen are not due to any change in the number of quantal events happening because of a presynaptic effect.

Perhaps the most interesting and direct piece of data to come out of this thesis is the relative membrane hyperpolarization without a change in EJP amplitude. Determining the exact mechanism by which this hyperpolarization is occurring would be the most direct way to develop a better working model of action for acetoacetate. There are many different ways this could be accomplished. Application of an agonist to the preparation could be another method to verify that there are postsynaptic effects. However, agonists are known to cause very rapid glutamatergic receptor internalization, which could make analysis of data very difficult. If a couple of ion channels were identified as candidates for causing the hyperpolarization, a blocker could be applied onto the preparation prior to the application of acetoacetate.

Alternatively, one could perform current-voltage curves and watch for the point at which the stepped current injection offsets the hyperpolarization. If this happened at a voltage that is at, or very close to, the equilibrium potential of an ion, one could determine which ion is fluxing and causing this change. Ionic concentrations in the saline bath could be systematically altered in order to determine which ion may be eliciting the effect. Following this, quantal event measurements could be done in the presence of a blocker for whatever channel is being affected to directly investigate the potential effects of acetoacetate on quantal events apart from the hyperpolarization. This could either validate the findings presented here or possibly show effects that were previously hidden.

As with any scientific finding, it is important to show that effects seen are able to be induced in many different contexts. This was done in the synaptic depression studies

by inducing ketosis via diet. However, there are other ways in which this could be done. Other neuromuscular junctions in other model organisms use glutamate, making them possible targets for validating results seen. Additionally, all of the assays completed for this thesis could be performed again in a dose-response manner with lithium acetoacetate or could even be performed with beta-hydroxybutyrate, the other major ketone body known to be produced in ketosis.

As with all studies, there are some possible shortcomings with this research. It is possible that the VGLUT at the *Drosophila* NMJ could be a different isoform in vertebrates. This could lead to some changes in the way that synaptic transmission could be modulated or even differences in its dependence on chloride for packaging. The hyperpolarization found could be an effect that is unique to muscles and not other neurons; it could even be specific for *Drosophila* muscles. Overall, the model is not as complex as other vertebrate models and lacks some components. For instance, there is no glia at the *Drosophila* NMJ. However, there are many attributes of this model that make it still a desirable organism for studying this phenomenon. Future research could look at confirming the data obtained from these experiments in vertebrate models.

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PROFESSIONAL PUBLICATIONS

1. Grau, E., Stanback, A.E., Bradley, A., Cantrell, D., Eversole, S., Grachen, C., Hall, K., Hawthorne, D., Kinmon, C., Ortiz Guerrero, P., Patel, B., Samuels, K., Suryadevara, C., Valdes, G., Wycoff, S., Cooper, R.L. (2018) Investigating the effects of homocysteine as an agonist on invertebrate glutamatergic synapses. IMPLUSE The Premier Undergraduate Neuroscience Journal. pp.1-12 https://impulse.appstate.edu/
2. Weineck, K., **Stanback, A.E.**, and Cooper, R.L. (2019) The effects of eugenol as an anesthetic for an insect: Drosophila, adult, larval heart rate and synaptic transmission. Article # In: McMahon K., editor. Tested studies for laboratory teaching. Volume 40. Proceedings of the 39th Conference of the Association for Biology Laboratory Education (ABLE). http://www.ableweb.org/volumes/vol-40/?art=#
3. **Alexandra E. Stanback**, Robin L. Cooper. The Effects of a Ketogenic Diet on Behavior and Synaptic Transmission in a Drosophila Model. (In preparation).
4. **Alexandra E Stanback**, Maddie Stanback, Allison Lane, Ross Basham, Bernardo Aguzzoli Heberle, Aaron Silverstein, Bennett Collis, Bharath Chithrala, Catherine Stanley, Emma Higgins, Kelsi Vela, Matthew Ponder, Prachi Raichur, Saadia Akhtar, Saisindhu Marella, and Robin L. Cooper. Effects of TEA and 4-AP on Firing Frequency of Proprioceptive Neurons in Crustaceans. (In preparation).
5. Stanback, M., **Stanback, A.E.,** Akhtar, S., Basham, R., Chithrala, B., Collis, B., Heberle, B.A., Higgins, E., Lane, A., Marella, S., Ponder, M., Raichur, P., Silverstein, A., Stanley, C., Vela, K. and Cooper, R.L. (2019). The effect of lipopolysaccharides on primary sensory neurons in crustacean models. IMPLUSE.

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